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(54) Title: SCREENING ASSAY

Simple morphology of NG2+ cells

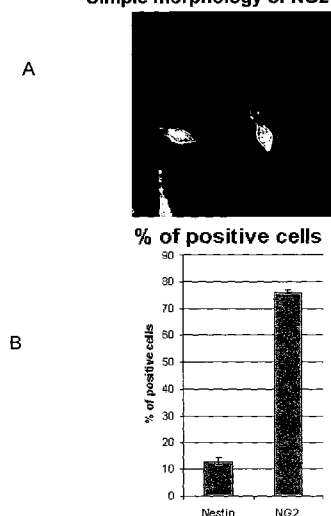


Figure 1

(57) **Abstract:** The present invention relates to a method of screening a candidate agent for the ability to stimulate differentiation of oligodendrocyte progenitor cells (OPCs) comprising i) contacting a cell with a candidate agent in the presence of a non-toxic amount of a cell division inhibitor and ii) determining if the candidate agent induces differentiation of the cell, wherein the OPC is a cell characterised as being capable of proliferating indefinitely. The invention further relates to candidate agents identified by the screening methods of the invention. Also included are methods of treating patients who suffer from or who are predisposed to suffer from Multiple Sclerosis (MS) or other demyelinating disorders.



Screening Assay

The present invention relates to a method of screening a candidate agent for the ability to stimulate differentiation of oligodendrocyte progenitor cells (OPCs) comprising i) contacting a cell with a candidate agent in the presence of a non-toxic amount of a cell division inhibitor and ii) determining if the candidate agent induces differentiation of the cell, wherein the OPC is a cell characterised as being capable of proliferating indefinitely. The invention further relates to candidate agents identified by the screening methods of the invention and to methods of treating patients who suffer from or who are predisposed to suffer from Multiple Sclerosis (MS) or other demyelinating disorders.

MS is a debilitating and chronic inflammatory disease of the central nervous system. In terms of natural history of MS, it is thought that most patients begin with relapsing and remitting symptoms and develop secondary progressive phase of disease following a number of relapse-remission divisions (Lancet, 2002, 359:1221-1231). A hallmark feature of MS pathology is demyelination of axons through inflammation-mediated damage of myelin-forming cells, oligodendrocytes (N Engl J Med, 2006, 354: 942). Remyelination, in response to demyelination, spontaneously occurs by the recruitment of OPCs into lesions and their subsequent differentiation into remyelinating oligodendrocytes (J Anat. 2005, 207: 251).

It has been suggested that this intrinsic repair of myelin via OPC differentiation is extensive during the relapsing-remitting phase and fails during the secondary progressive phase resulting in persistent demyelination (Brain, 2006, 129: 3165 and Nat Rev Neurosci, 2002, 3: 705). Although it remains unknown at what stage and why the repair process fails, a significant population of OPCs is suggested to be present in MS lesions (Brain, 2008, 131: 1749; N Engl J Med, 2002, 346: 165 and Nat Rev Neurosci, 2002, 3: 705). These progenitor cells would be of therapeutic value if their controlled conversion into remyelinating oligodendrocytes was possible.

The method of the invention provides an *in vitro* cell-based system that is suitable for high throughput screening in order to identify agents that can induce OPC

differentiation into mature oligodendrocytes. The method includes the benefits of (1) easily obtainable biomass of cells, (2) reliable parameters for OPC differentiation during a short period of culture and (3) robust signal-to-noise ratio (i.e. the effect of pharmacologic reagents in comparison to the control is clearly determinable).

5

The first aspect of the invention is a method of screening a candidate agent for the ability to stimulate differentiation of oligodendrocyte progenitor cells (OPCs) comprising i) contacting an OPC with a candidate agent in the presence of a non-toxic amount of a cell division inhibitor and ii) determining if the candidate agent induces
10 differentiation of the OPC, wherein the OPC is a cell characterised as being capable of proliferating indefinitely.

The OPC of the invention is a cell which is an oligodendrocyte progenitor cell that proliferates indefinitely (i.e. it is an immortalised oligodendrocyte cell). The OPC of
15 the invention is likely to have its immortal properties as a result of a genetic modification or alteration. The genetic modification or alteration may be spontaneous or induced. The genetic modification or alteration may be such that the cell has a constitutive mitogenic signal which causes the immortal property of the cell. The OPC of the method of the invention is preferably part of a population of OPCs.

20

The Oli-neu cell line is a known cell line that constitutes a homogeneous population of OPCs that express markers such as NG2+ (an OPC marker) (Eur. J. Neurosci., 1995, 7: 1245-65, J. Neurol. Sci., 2005, 233: 37 and Nat. Rev. Neurosci., 2002, 3: 705). Oli-neu cells are made by immortalising primary mouse OPCs by infection with retroviruses
25 expressing the t-neu oncogene. Transforming OPCs with the t-neu oncogene constitutively activates erbB2-associated tyrosine kinase. The activated erbB2 induces unlimited expansion of OPCs providing enough cell mass for high throughput screening, while maintaining their progenitor nature.

30 It has been reported that Oli-neu cell differentiation is induced by 10-day treatment with membrane permeable cAMP analogue dibutyryl cAMP (dbcAMP) (Eur. J. Neurosci., 1995, 7: 1245-65). In the absence of dbcAMP, Oli-neu cells are of simple morphology. The treatment with dbcAMP induces Oli-neu cell differentiation into myelin associated

glycoprotein (MAG)-expressing cells which exhibit complex morphology such as extension of cell processes.

5 The Epidermal Growth Factor (EGF) or the ErbB family of receptor tyrosine kinases consists of four members: EGF receptor/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. Under normal physiological conditions, the ErbB receptors play crucial roles in cell proliferation, differentiation, motility, and apoptosis. The ErbB proteins are also associated with human tumorigenesis. The ErbB family members are receptors for Neuregulins (NRGs) and EGF family growth factors. Upon
10 ligand binding, ErbB receptors form homo- and heterodimers leading to the activation of their tyrosine kinase domain and subsequent phosphorylation of tyrosine residues in the cytoplasmic tail. ErbB2 is an orphan receptor and requires heterodimer formation with a different ligand-bound family member to become activated (Journal of Molecular and Cellular Cardiology. 2008; 44:831-854).

15 OPC differentiation in accordance with the invention means differentiation of OPCs into mature oligodendrocytes. OPC differentiation can be determined morphologically or using a gene-expression reporter assay. The advantage of a morphology-based screening approach is evident from pathological evidence in MS (N Engl J Med, 2002, 20 346: 165; Brain, 2008, 131: 1749) and in animal models of MS. Persistently demyelinated lesions are characterised by abundantly recruited OPCs but their differentiation is impaired at the premyelinating oligodendrocyte stage of development. These cells showed simple morphology with less extended cell processes. This pathology data implicates enhancement of oligodendrocyte process extension as a
25 therapeutic parameter.

Differentiation according to the invention can be associated with an increase in markers of late stage of oligodendrocyte differentiation, such as the myelin basic protein (MBP) marker and/or the MAG marker in the bodies and/or an increase in the cell processes.

30 OPC differentiation can be quantified visually by identifying one or more late oligodendrocyte markers and/or extension of cell processes positive for said late oligodendrocyte marker using phase contrast or fluorescence microscopy.

Any method to quantify OPC differentiation known in the art can be used. For example, methods are known for measuring fluorescence intensity of a mature oligodendrocyte marker signal and extension of cell processes positive for said mature
5 oligodendrocyte marker. For example, the Acumen eX3 microplate cytometer (TTP LabTech Ltd) can be used to measure marker signal and the Cellomics ArrayScan High Content Screening Reader (Thermo Fisher Scientific Cellular Imaging) can be used to measure extension of cell processes positive for a particular marker.

10 Methods of screening using the Oli-neu cell line are known but such methods are considered not to be highly sensitive. Thus, there is a requirement for more sensitive methods of identifying therapeutic agents that stimulate OPC differentiation into mature oligodendrocytes, as such agents would be useful for treating MS and other demyelinating disorders.

15

Thus, the inventors have identified that by contacting an OPC, which is a cell characterised as being capable of proliferating indefinitely, with a cell division inhibitor, it is possible to increase the sensitivity of the cell to differentiation inducing agents. This novel finding can be utilised in the method of the invention to provide a more
20 sensitive screen for agents that induce cell differentiation.

The candidate agent in accordance with the invention can be any natural or synthetic molecule, for example small chemical molecules, proteins or nucleic acids (including siRNA molecules). Preferably the candidate agent is a small molecule. The candidate
25 agent can be a molecule which is known or it can be a novel molecule.

The method of the invention can optionally include comparison of differentiation of an OPC of the invention that has been contacted with both a candidate agent and a cell division inhibitor against differentiation of an OPC that has been contacted with a cell
30 division inhibitor only, i.e. a control. Any cell division inhibitor that blocks indefinite proliferation, i.e. blocks the constitutive mitogenic signalling pathway, causing a statistically significant induction of cell differentiation at a non-toxic concentration can be used as a positive control. The OPC of the present invention is likely to be

immortalised by a mutation in one or more parts of the ErbB family. Thus, in one embodiment, an erbB receptor inhibitor can preferably be used as a positive control in the method of the invention. The positive control can be used as a marker to confirm the cells' ability for differentiation in every assay. Preferably the erbB receptor inhibitor is an ErbB2 receptor inhibitor, such as TKi-39 (N-(4-((3-Chloro-4-fluorophenyl) amino)pyrido[3,4-d]pyrimidin-6-yl)2-butyramide, Merck Chemicals Limited, #324840), 4557W (4-(4-Benzoyloxyanilino)-6,7-dimethoxyquinazoline, Merck Chemicals Limited, #324673) or Iressa (Gefitinib, LC Laboratories, G-4408).

10 The method of the invention includes the addition of a non-toxic amount of a cell division inhibitor that can enhance assay sensitivity and the addition of a non-toxic amount of a cell division inhibitor that causes a statistically significant induction of OPC differentiation to be used as a positive control.

15 Typically, the non-toxic amount of a cell division inhibitor that is utilised to enhance assay sensitivity is a "low" amount, preferably it is the lowest amount that shows a significant induction of differentiation above background levels. More preferably the amount of cell division inhibitor is an amount that provides the threshold of response with minimal induction of differentiation.

20

The non-toxic amount of cell division inhibitor used in the positive control is an amount that results in statistically significant induction of OPC differentiation. Typically a "high" amount of the cell division inhibitor is used, preferably it is an amount that causes a large, clearly above threshold induction of OPC differentiation. More preferably, it causes around maximal achievable amount of OPC differentiation with said cell division inhibitor.

25

The cell division inhibitor used to enhance assay sensitivity can be the same or a different cell division inhibitor to the one used as the positive control.

30

A cell division inhibitor according to the invention is any agent that is capable of inhibiting cell division of the OPC of the invention at a concentration that does not cause cell toxicity. A cell division inhibitor according to the invention is capable of

inhibiting cell division but does not necessarily inhibit cell division when used in the method of the invention.

- Whether or not an agent is capable of inhibiting cell division can be determined by incubating cells with an increasing concentration of an agent and quantifying the number of cell nuclei stained with Hoechst 33342 using Cellomics ArrayScan High Content Screening Reader. If cell division is inhibited in a dose dependent manner, the agent is a cell division inhibitor.
- 10 Whether or not the cell division inhibitor of the invention inhibits cell division when used in the method of the invention will depend on the concentration of cell division inhibitor used. The particular concentration at which inhibition of cell division no longer occurs will depend on the particular cell division inhibitor used.
- 15 A cell division inhibitor when used in the method of the invention has a positive effect on OPC differentiation. There is a relationship between cell differentiation and cell division and the decrease in cell division is associated with an increase in cell differentiation.
- 20 A non-toxic amount of a cell division inhibitor used in the method of the invention is an amount that does not kill the OPC to which the cell division inhibitor is administered. Preferably the amount of cell division inhibitor maximises the signal to noise ratio. The signal to noise ratio is the ratio of the amount of differentiation when an OPC is treated with both a cell division inhibitor and a further candidate agent that induces
- 25 differentiation relative to the level of differentiation when an OPC is treated with a cell division inhibitor only. Preferably, the amount of cell division inhibitor is the lowest amount that shows a significant induction of differentiation above background levels, more preferably the amount of cell division inhibitor is an amount that provides the minimal threshold of induction of differentiation. Typically, the amount of the cell
- 30 division inhibitor may be less than 50%, preferably less than 40%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, less than 3%, less than 2% or even less than 1% of the non-toxic amount of cell division inhibitor that

would induce a maximal differentiation response for the particular cell division inhibitor in the OPC.

5 It will be appreciated that the amount of cell division inhibitor used will depend on the type of cell division inhibitor that is used. The amount of cell division inhibitor can be at least 7nM, between about 7nM and 1000nM or between about 60nM and 800nM, for example. When the cell division inhibitor is TKi-39, the preferred amount is about 7nM or more, more preferably the amount is about 20 to 25nM. The skilled person will be able to determine the amount of cell division inhibitor by standard titration experiments.

10

Any cell division inhibitor can be used in the method of the invention. For example, inhibitors of DNA synthesis can be used such as 5-Fluorouracil (5FU); a potent anti-tumour agent that affects pyrimidine synthesis by inhibiting thymidylate synthetase. Treatment of cells with 5FU leads to an accumulation of cells in S-phase and has been
15 shown to induce p53 dependent apoptosis (Biochem. Pharmacol., 1998, 55:1353-1360). Cisplatin; a platinum coordination complex with potent anti-neoplastic activity that induces apoptosis in cancer cells (Neurooncol., 2001, 52:23) and Etoposide; a cell-permeable derivative of podophyllotoxin that acts as a topoisomerase II inhibitor and has major activity against a number of tumours (J. Virol., 1995, 69:2082). Inhibitors of
20 mitogenic signals can also be used such as erbB-associated tyrosine kinase inhibitor (TKi-39), which is a cell-permeable, ATP-binding site-targeting alkynamidopyrimidine compound that acts as a potent and irreversible inhibitor of erbB activities (J. Med. Chem., 2006, 49:1475). Iressa and 4557W are further examples of cell division inhibitors that can be used in the method of the invention.

25

The cell division inhibitor and candidate agent can be simultaneously incubated with the OPCs of the invention.

30 A second aspect of the invention relates to a method of screening a candidate agent for the ability to stimulate differentiation of OPCs comprising: (i) contacting an OPC with a candidate agent in the presence of a non-toxic amount of a cell division inhibitor, (ii) a positive control that comprises contacting an OPC with a non-toxic amount of a cell division inhibitor in the absence of a candidate agent, (iii) comparing OPC

differentiation of the positive control with OPC differentiation in the presence of the candidate agent, and (iv) determining if the candidate agent induces differentiation of the OPC, wherein the OPC is characterised as being capable of proliferating indefinitely. In this method, the OPC that is contacted with the candidate agent is a
5 separate population of OPCs to the population of OPCs that is contacted with a cell division inhibitor in the absence of a candidate agent. The cell division inhibitor used in step (i) and the cell division inhibitor used in step (ii) may be the same cell division inhibitor or different cell division inhibitors. Preferably the cell division inhibitor used in the positive control is an erbB2 inhibitor. The non-toxic amount of cell division
10 inhibitor in step (i) is preferably a "low" amount, whereas the non-toxic amount of cell division inhibitor in step (ii) is preferably a "high" amount (see above).

A third aspect of the invention relates to an agent which induces differentiation of OPCs into mature oligodendrocytes and which is identified by the method of the first aspect of
15 the invention. Such an agent can be in combination with a pharmaceutically acceptable excipient. Such a combination can be referred to as a pharmaceutical composition. Such a pharmaceutical composition can be used to treat MS (e.g., Relapsing-Relapsing MS, Secondary Progressive MS, Primary Progressive MS, and Progressive-Relapsing MS) and other demyelinating disorders. A demyelinating disorder is any disease
20 associated with the damage of myelin sheath insulating axons, or injury involving demyelination or dysmyelination. Demyelinating disorders include, for example, Chronic Inflammatory Demyelinating Polyneuropathy, Inherited Neuropathies, Alexander disease, Guillain-Barre syndrome, Acute Disseminated Encephalomyelitis, Subacute Sclerosing Panencephalitis, Post-infectious Encephalomyelitis, Devic's
25 Disease, Balo's Concentric Sclerosis, Adrenomyeloneuropathy, Adrenoleukodystrophies, Krabbe's disease, Metachromatic Leukodystrophy, Pelizaeus-Merzbacher disease, Tay-Sachs disease, Niemann-Pick disease, Gaucher's disease, Canavan disease, Phenylketonuria, Childhood Ataxia with Central Hypomyelination, Alexander disease, Hurler's syndrome, Refsum disease, Leber's hereditary optic
30 atrophy, Spinal Cord Injury, Traumatic Brain Injury, Post Radiation Injury, Stroke, Carbon Monoxide Toxicity, Syndromes of Delayed Hypoxic Cerebral Demyelination, Progressive Subcortical Ischemic Demyelination, Central Pontine Myelinolysis, Demyelination of the corpus callosum (Marchiafava-Bignami disease), Vitamin B12

deficiency, Progressive Multifocal Leukoencephalopathy, Subacute Sclerosing Panencephalitis, Tropical spastic paraparesis/ human T-lymphotropic virus 1-associated myelopathy, Optic Neuritis, Acute Transverse Myelitis, Acute Disseminated Encephalomyelitis, Acute Hemorrhagic Leukoencephalitis, Neuromyelitis Optica, 5 Infectious Demyelinating Diseases, Protein Inclusion Diseases (Alzheimer's disease, Parkinson's disease, advanced Down's syndrome, Prion diseases, Huntington's disease, spinocerebellar ataxias, serpinopathies). Preferably the demyelinating disorder is optical neuritis or spinal cord injury. Most preferably, the demyelinating disorder is MS.

10

A fourth aspect of the invention relates to a method of treating a patient who suffers from or who is predisposed to suffer from MS or other demyelinating disorders, the method comprising screening a candidate agent according to the first aspect of the invention, identifying an agent that induces differentiation of OPCs and administering 15 the agent to the patient. Preferably, the patient is in need of treatment for MS or another demyelinating disorder. Treatment can be therapeutic or prophylactic.

A fifth aspect of the invention provides the agent according to the third aspect of the invention for use in treating MS and other demyelinating disorders. A sixth aspect 20 provides the use of the agent according to the third aspect of the invention in the manufacture of a medicament for treating MS and other demyelinating disorders. A seventh aspect of the invention provides a pharmaceutical composition comprising the agent according to the third aspect of the invention.

25 All preferred embodiments of the first to seventh aspects of the invention, also apply to each other aspect, *mutatis mutandis*.

The present invention is described with reference to the following figures and tables, in which:

30

Figure 1 illustrates the progenitor nature of undifferentiated Oli-neu cells. A) Simple morphology of NG2+ cells (bipolar). B) Quantification of Nestin+ and NG2+ cells in undifferentiated Oli-neu cells by Cellomics. Mean $\pm$ SD (n=4);

Figure 2 illustrates detection of MBP positive cells by Cellomics. Circles indicate MBP+ cells with complex morphology, x200. Two circles are drawn 7.7 μ m and 9.7 μ m from the nuclei boundaries (centre of the circles) creating two rings. The inner ring (ring1) contains the cell body and the outer ring (ring2) contains the cell processes;

Figure 3 illustrates induction of Oli-neu cell differentiation by dbcAMP. A) Quantification of Hoechst 33342+ in control vs dbcAMP (1mM) treated. B) Quantification of Oli-neu cell differentiation by calculating the ratio of MBP+ cells with extended processes to total number of Hoechst 33342+ nuclei. Mean $\pm$ SD (n=4), *p<0.01 (t-test);

Figure 4 illustrates failure in induction of Oli-neu cell differentiation by T3 and CPT-cAMP. T3 (300ng/ml) and CPT (50 μ M). Mean $\pm$ SD (n=4), p>0.05 (t-test);

Figure 5 illustrates induction of Oli-neu cell differentiation through inhibiting cell division. A) Inhibition of cell proliferation as quantified by number of Hoechst 33342+ nuclei in 10 fields. B) Induction of cell differentiation as quantified by %MBP+ cells (MBP+ cell number/Hoechst 33342+ nucleus number). Mean $\pm$ SD (n=4) * control versus treated groups, p<0.01 (t-test);

Figure 6 illustrates elicited efficacy of T3 and CPT-cAMP under the inhibition of cell division. T3 and CPT-cAMP in the presence of various concentrations (22, 66, 200, 600nM) of cell division inhibitors (Cisplatin (A), 5FU (B), Etoposide (C), TKi-39 (D)) showed 2- to 5-fold induction of differentiation. *control versus T3 in the presence of individual concentration of cell division inhibitors. Mean $\pm$ SD (n=4) (p<0.01, t-test); #control versus CPT-cAMP in the presence of individual concentration of cell division inhibitors. Mean $\pm$ SD (n=4) (p<0.01, t-test) and

Figure 7 illustrates elicited efficacy of T3 in the presence of TKi-39 at 22nM. T3 (300ng/mL), mean $\pm$ SD (n=4) *control versus T3 in the presence of 22nM TKi-39. Mean $\pm$ SD (n=4) (p<0.01, t-test).

Table 1 illustrates a list of various inhibitors of cell division used in the Oli-neu cell culture and

Table 2 illustrates upregulated efficacy of pharmacologic reagents in the presence of
5 TKi-39 at 22nM relative to that in the absence.

The invention will now be described by way of reference to the following Examples, which are provided for the purposes of illustration only and are not to be construed as being limiting to the invention.

10

Examples

The cell line from which the OPCs of the invention are obtained may also be characterised in that at least 70% of the cells are NG2+. Preferably the cells of the invention are obtained from the Oli-neu cell line (Eur. J. Neurosci., 1995, 7:1245-65).

Example 1 Oli-neu cell differentiation

Method

Oli-neu cell culture: Oli-neu cells were obtained from Prof. Jacqueline Trotter, Johannes Gutenberg-Universität Mainz). In brief, the Oli-neu cells are made by immortalising primary mouse OPCs by infection with retroviruses expressing the t-neu oncogene. Transforming t-neu constitutively activates erbB2-associated tyrosine kinase. The activated erbB2 induces unlimited expansion of OPCs providing enough cell mass for high throughput screening, while maintaining their progenitor nature (Eur. J. Neurosci., 1995, 7: 1245-65).

The majority of Oli-neu cells are characterised by the expression of OPC marker NG2+ (>75%), whereas a small proportion expresses the neural stem cell marker Nestin (<15%) (Figure 1).

The Oli-neu cells were grown in a 384 well poly-L-lysine (PLL)-coated plates (BD biocoat Cat number 354663) at 750 cells per well in DMEM (ATCC, 30-2002) containing B27 (Invitrogen, 17504-044), N2 (Invitrogen, 17502-048) supplements, 2g/L NaHCO₃, 1% horse serum (ATCC, 30-2040) and Genamycin (50mg/ml). Cells were treated for 3 days with dbcAMP (1mM), Thyroid hormone triiodothyronine (T3) (Sigma, T6397) (300ng/ml) or cAMP analogue 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) (Sigma, C3912) (50uM). dbcAMP induces Oli-neu cell line differentiation and T3 and CPT-cAMP are known to induce OPC differentiation in primary OPC culture (Dev. Neurosci., 1985, 7: 45-54 and J. Neurosci., 1998, 18: 4627-4636).

Immunostaining and data analysis: Cells were fixed with 3.2% paraformaldehyde (PFA) for 15 min, washed once with PBS, then immunostained with custom-made

fluorescently-labelled rat anti-MBP antibody (Serotec, MCA409S) at 40ng/well. Nuclei were stained with Hoechst 33342 (Sigma, B 2261). MBP-expressing cells were quantified by measuring %MBP+ cells with extended processes (number of MBP+ cells with extended processes to total number of Hoechst 33342+ cells). As shown in Figure 2, presence of MBP positive cells was determined by drawing two circles around a cell nuclei, 7.7µm and 9.7µm (7.7µm and 15.8µm with 100X objective) from the nuclei boundaries, creating two rings. The inner ring contains the cell body and the outer ring includes the cell processes. A positive cell is determined as follows: 1) the intensity of a cell body (inner ring) is above background threshold and 2) the intensity of processes (outer ring) is above a certain threshold that is determined visually in every experiment. For this purpose, Cellomics ArrayScan compartmental analysis function (Thermo Fisher Scientific Cellular Imaging) was utilised by selecting cells with certain signal intensity outside cell body.

15 Results

Oli-neu cell differentiation is induced by 10-day treatment with 1mM dbcAMP (Eur. J. Neurosci., 1995, 7: 1245-65). The Oli-neu cells treated for 3 days with 1mM dbcAMP showed 2.3-fold induction of differentiation without affecting cell number (Figure 3), as determined by the morphology-based high content method.

20 Neither 300ng/ml T3 (a thyroid hormone) nor 50µM CPT-cAMP (a cAMP analogue) showed significant effect on Oli-neu cell differentiation compared to control in 3 day culture (Figure 4).

25 Example 2

Method

Oli-neu cells were grown as described above in example 1 but in the presence of the inhibitors of cell division set out in table 1 rather than in the presence of dbcAMP, T3 or CPT-cAMP. The Oli-neu cells were then analysed, also as discussed above in Example 1.

Results

Oli-neu cells treated with the inhibitors of cell division set out in table 1 (5FU, Cisplatin, Toposide or TKi-39) showed inhibition of proliferation that coincided with induction of differentiation in a dose-dependent manner (Figure 5). TKi-39, Cisplatin, 5FU and Etoposide all induced differentiation. Cisplatin, 5FU and Etoposide caused 2-
5 fold induction of differentiation at the highest concentration (600nM). TKi-39 significantly induced cell differentiation by 2-fold at the lowest concentration (22nM) with the most extensive induction of differentiation at 600nM (by 5-fold).

Example 3

10

Method

Oli-neu cells were incubated as described above in Example 1 in the presence of T3 or CPT-cAMP and the cell division inhibitors set out in table 1. The Oli-neu cells were then analysed, also as discussed above in Example 1.

15

Results

Treatment of Oli-neu cells with T3 (300ng/ml) or CPT-cAMP (50µM) in the presence of various cell division inhibitors elicited 2- to 5-fold induction of differentiation compared to cells treated with control (cell division inhibitors only) (Figure 6). This
20 indicates that the addition of cell division inhibitors in the assay medium increases the sensitivity of the assay to elicit the effect of T3 and CPT-cAMP. Among these inhibitors, TKi-39 provides the most robust signal-to-noise ratio (by 5-fold induction for 22nM TKi-39+CPT-cAMP, Figure 6D).

25 Further titration of TKi-39 has shown that 22nM exhibited an optimum increase in the assay sensitivity to elicit the effect of T3 (Figure 7). This may be because 22nM TKi-39 constitutes the threshold of response with minimal induction of differentiation (2-fold increase in %MBP+ cells from 4% to 8%, Figure 5B), allowing a window of response for other treatments such as T3 (3.5-fold increase in %MBP+ cells from 8% to 28%
30 with T3, Figure 6D). Other concentrations of TKi-39 lower than 22nM had no effect on increasing assay sensitivity to elicit the effect of T3 (Figure 7). TKi-39 concentrations higher than 22nM caused a potent induction of differentiation resulting in a narrower window of response to T3 (1.4-fold increase in %MBP+ cells from 40% to 55%, Figure

6D). Thus, including TKi-39 at 22nM in the assay medium provided the most robust signal-to-noise ratio with T3 treatment.

Example 4

5

Method

The effects of the pharmacological agents such as thyroid hormone (T4, Sigma # T2501), Gamma-secretase inhibitor (DAPT, Merck Chemicals Limited, # 565784), PI3 Kinase inhibitor (LY294002, Cell signalling, # 9901), cAMP analogue (Forskolin, 10 Sigma # F6886) and erbB tyrosine kinase inhibitor (Iressa, LC Laboratories # G-4408) on Oli-neu cell differentiation were examined in the presence or absence of 22nM TKi-39. The Oli-neu cells were then analysed, also as discussed above in Example 1.

Results

15 All treatments showed greater effects in the presence of TKi-39, showing that TKi-39 at 22nM increases the assay sensitivity to elicit efficacy of the compounds that induce Oli-neu cell differentiation (see table 2; "level of response" indicates fold induction of differentiation over the DMSO control). The results from testing T3 and CPT-cAMP in example 3 are also included in table 2.

20

Cell division inhibitor	Mechanism of action
5FU (Fluorouracil), Sigma #F6627	DNA synthesis inhibitor
Cisplatin, Merck, #232120	DNA synthesis inhibitor
Etoposide, Merck, #341205	Topoisomerase II inhibitor
Tki-39, Merck, #324840	Inhibitor of erbB activities (EGFR, erbB-2, and erbB-4).

5

Table 1

	Absence of Tki-39	Presence of 22nM Tki-39
Candidate agent	Level of response compared to DMSO control	Level of response compared to DMSO control
DMSO control	1	1
Triiodothyronine (T3)	1.2	1.6
Thyroxine (T4)	1.1	1.5
DAPT	1	2.6
LY294002	2.5	3.1
CPT-cAMP	1.1	3.1
Forskolin	1.7	3.5
Iressa	5.6	5.8

10

Table 2

Claims

1. A method of screening a candidate agent for the ability to stimulate differentiation of oligodendrocyte progenitor cells (OPCs) comprising i) contacting an
5 OPC with a candidate agent in the presence of a non-toxic amount of a cell division inhibitor and ii) determining if the candidate agent induces differentiation of the OPC, wherein the OPC is a cell characterised as being capable of proliferating indefinitely.
2. The method of claim 1, wherein the candidate agent is a small molecule.
- 10 3. The method of claim 1 or claim 2, wherein the OPC is from an Oli-neu cell line.
4. The method of any one of claims 1 to 3, wherein the differentiation is determined by measuring a morphological change of the OPC.
- 15 5. The method of any one of claims 1 to 4, wherein the cell division inhibitor is an erbB inhibitor.
6. The method of any one of claims 1 to 5, wherein in addition, there is a positive
20 control comprising i) contacting the OPC in the presence of a non-toxic amount of a cell division inhibitor in the absence of a candidate agent and ii) comparing OPC differentiation of the positive control with OPC differentiation in the presence of the candidate agent.
- 25 7. A method of screening a candidate agent for the ability to stimulate differentiation of OPCs comprising:
 - (i) contacting an OPC with a candidate agent in the presence of a non-toxic amount of a cell division inhibitor,
 - (ii) a positive control that comprises contacting an OPC with a non-toxic
30 amount of a cell division inhibitor in the absence of a candidate agent,
 - (iii) comparing OPC differentiation of the positive control with OPC differentiation in the present of the candidate agent, and
 - (iv) determining if the candidate agent induces differentiation of the OPC,

wherein the OPC is characterised as being capable of proliferating indefinitely.

8. The method of claim 6 or claim 7, wherein the cell division inhibitor used in the positive control is an erbB2 inhibitor.

5

9. A candidate agent identified by the method of any one of claims 1 to 8.

10. A method of treating a patient who suffers or is predisposed to suffer from Multiple Sclerosis (MS) or other demyelinating disorder, the method comprising
10 screening a candidate agent as claimed in any one of claims 1 to 8, identifying an agent that induces differentiation of the OPCs and administering the agent to the patient.

11. An agent identified by the method of any one of claims 1 to 8 for use in treating MS or other demyelinating disorder.

15

12. Use of an agent identified by the method of any one of claims 1 to 8 in the manufacture of a medicament for treating MS or other demyelinating disorder.

13. The method, agent or use of claims 10, 11, or 12 respectively, wherein the
20 demyelinating disorder is selected from optic neuritis and spinal cord injury.

14. A pharmaceutical composition comprising an agent identified by the method of any one of claims 1 to 8.

Simple morphology of NG2+ cells

A



B

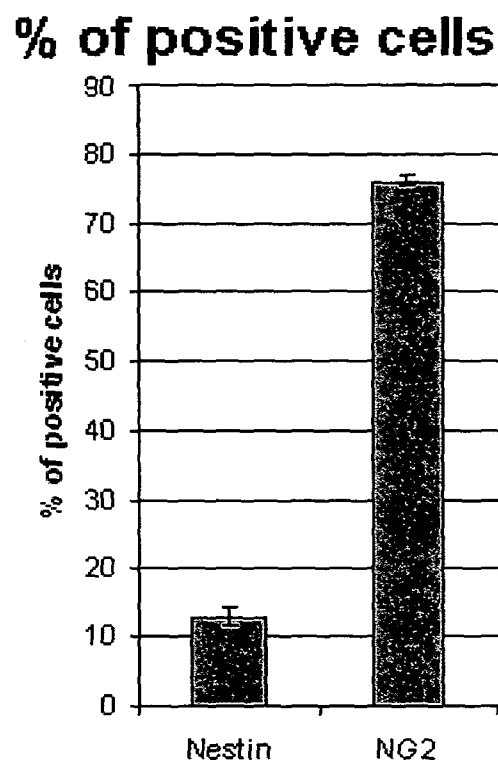


Figure 1

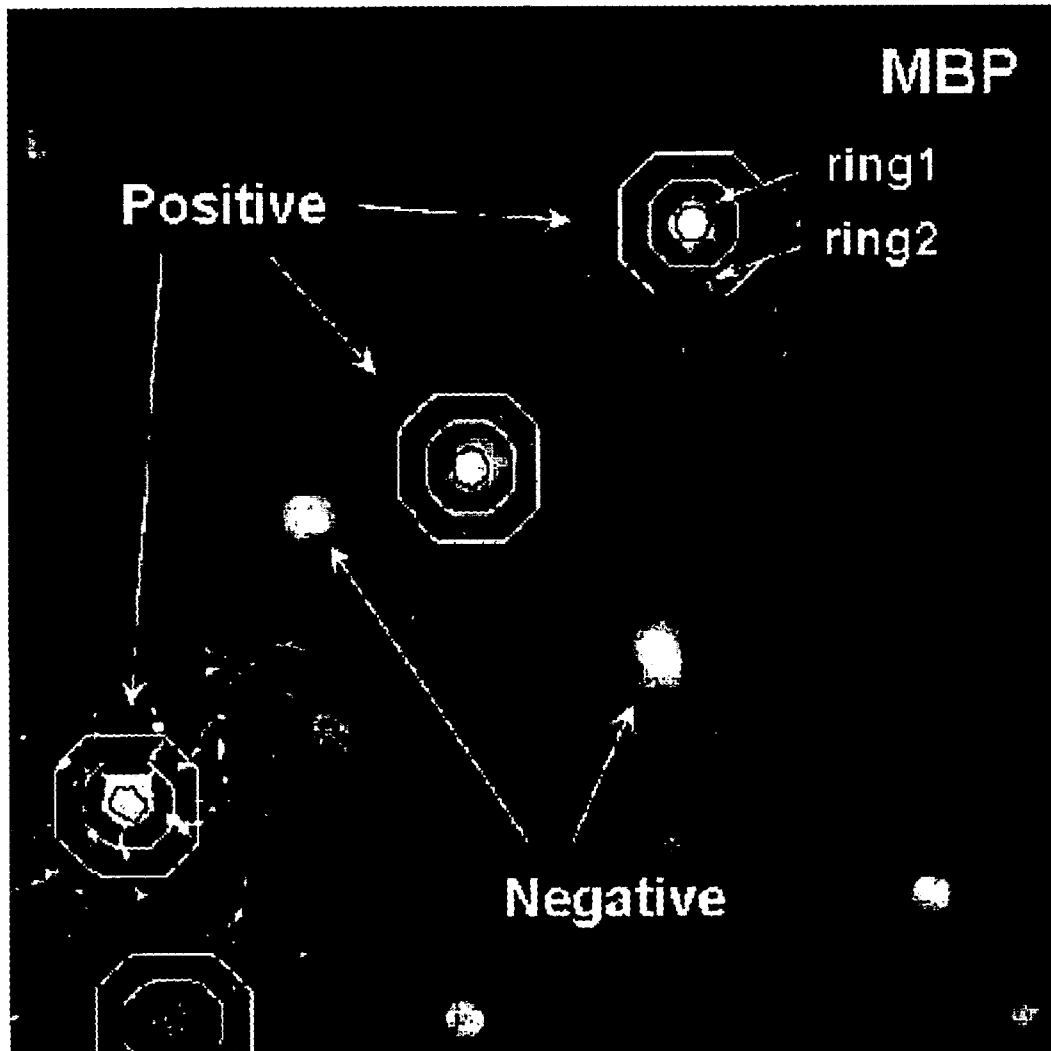


Figure 2

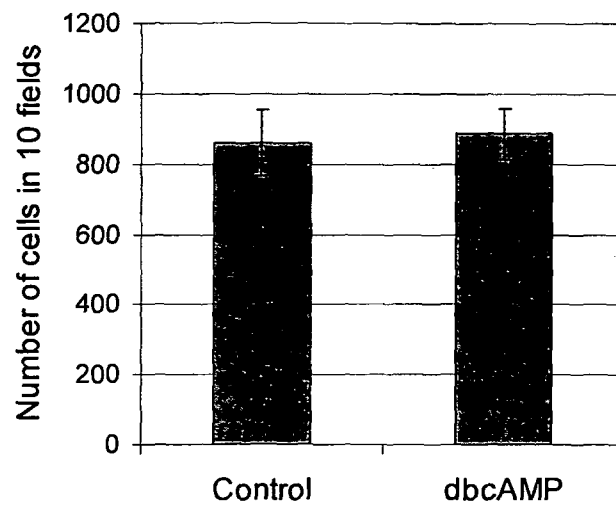
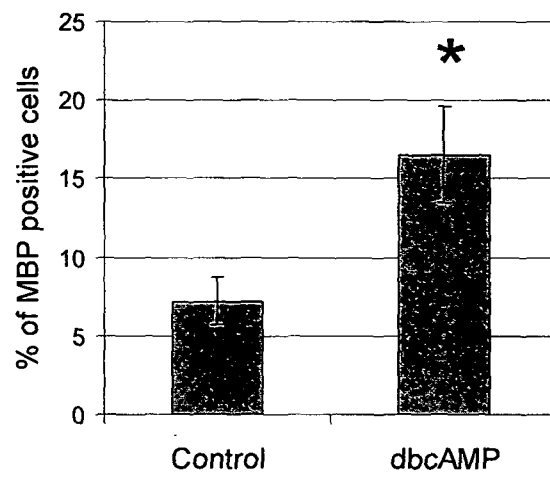
A**B**

Figure 3

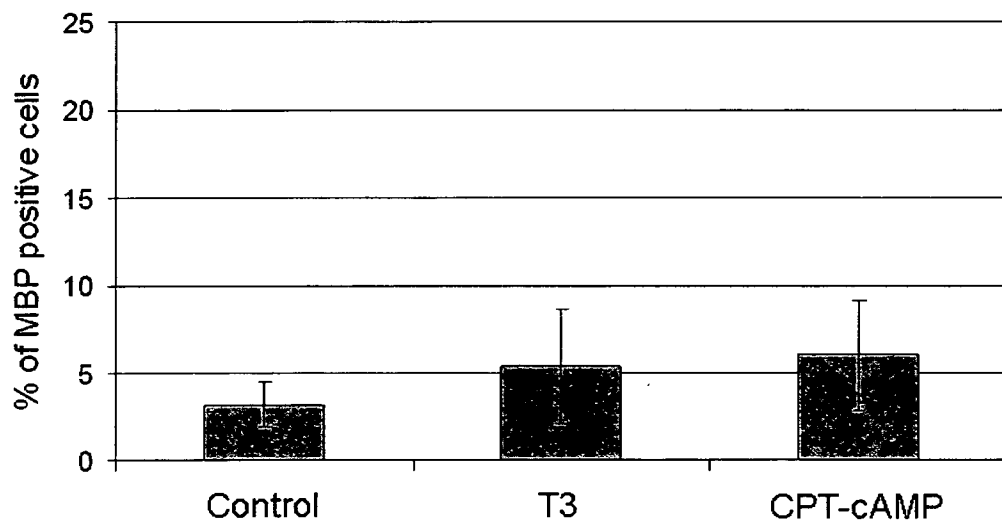


Figure 4

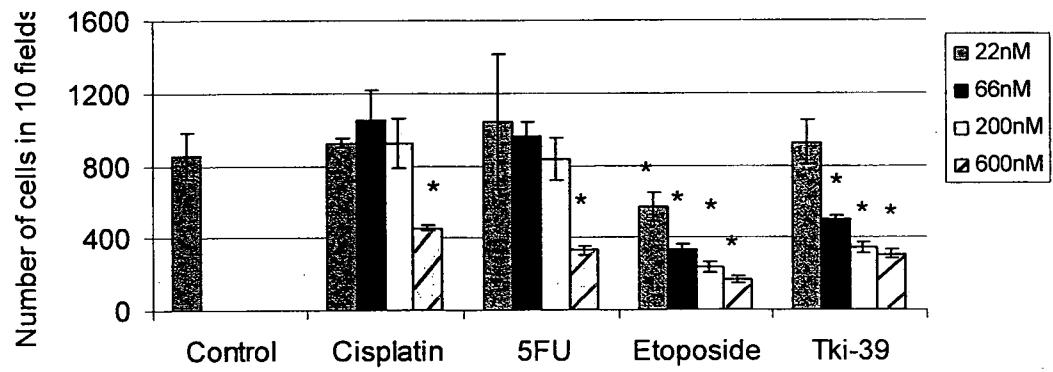
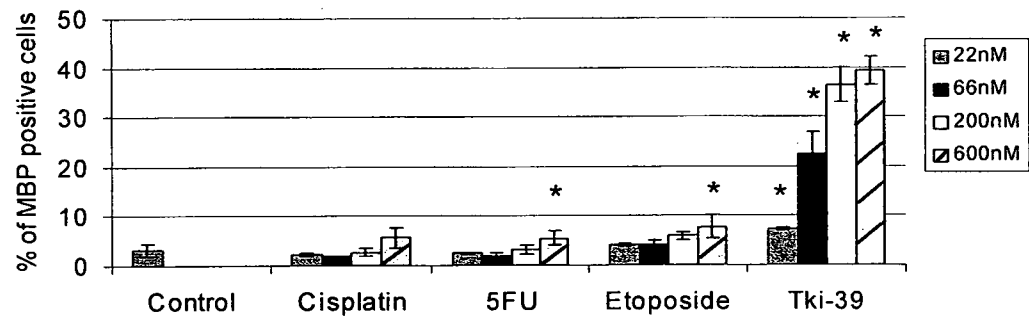
A**B**

Figure 5

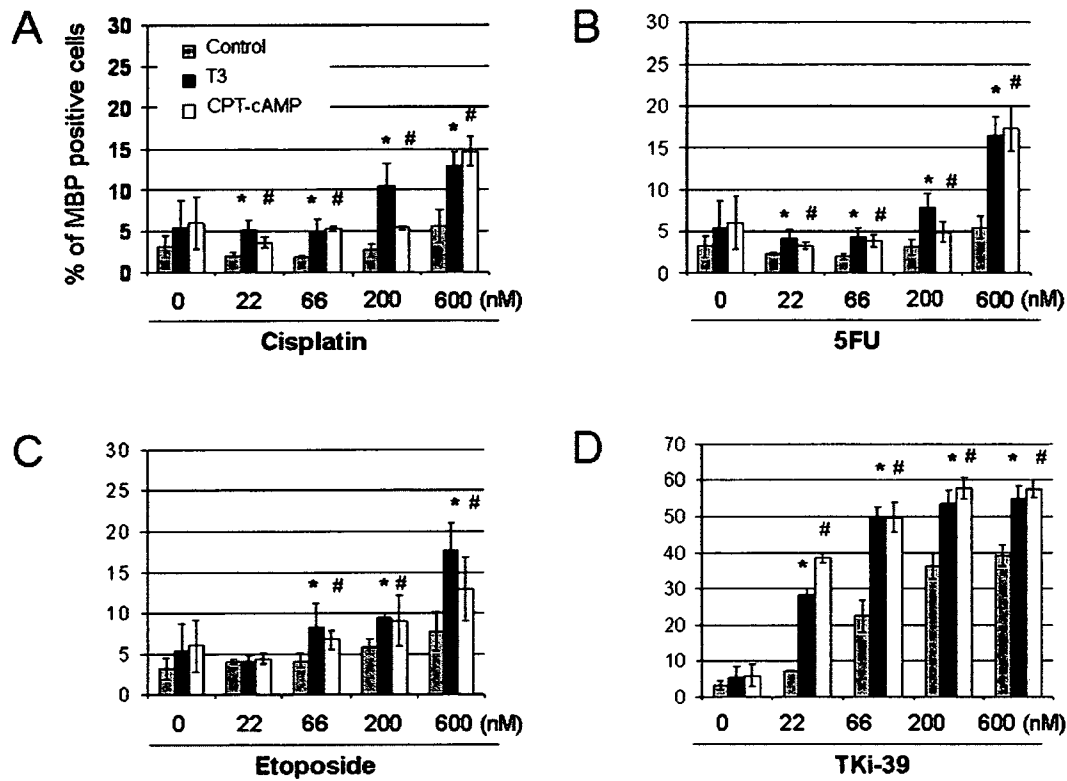


Figure 6

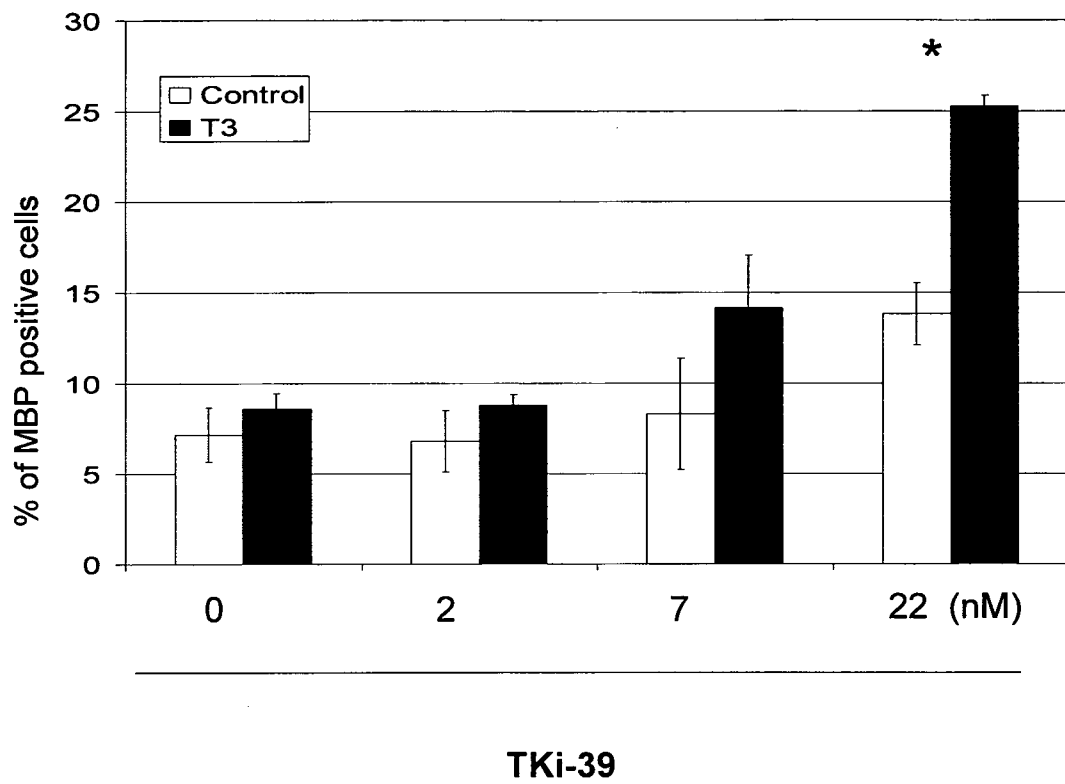


Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/002036

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50

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)
G01N

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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96/09543 A1 (NEUROSPHERES LTD [CA]) 28 March 1996 (1996-03-28) claim 10	1-8
A,P	ARIEN-ZAKAY H ET AL: "Interferon-gamma-induced neuronal differentiation of human umbilical cord blood-derived progenitors." LEUKEMIA, vol. 23, no. 10, 2009, pages 1790-1800, XP002554201 figures 4,7	1-8

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *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.
- *G* document member of the same patent family

Date of the actual completion of the international search

11 November 2009

Date of mailing of the international search report

04/12/2009

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Authorized officer

Gunster, Marco

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2009/002036

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 9-14
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:
see FURTHER INFORMATION sheet PCT/ISA/210
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 all searchable 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 report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 9-14

Present claims 9-14 encompass compounds defined only by their desired function, i.e. that they have been identified by the screening method of claim 1-8, contrary to the requirements of clarity of Article 6 PCT, because the result-to-be-achieved type of definition does not allow the scope of the claim to be ascertained. The fact that any compound could be screened does not overcome this objection, as the skilled person would not have knowledge beforehand as to whether it would fall within the scope claimed. Undue experimentation would be required to screen compounds randomly. The non-compliance with the substantive provisions is to such an extent that no meaningful search of claims 9-14 could be carried out at all (Article 17(2) PCT).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2009/002036

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9609543	A1	28-03-1996	
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		AT 208495 T	15-11-2001
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		ES 2167461 T3	16-05-2002
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		NO 971245 A	18-03-1997
		PT 783693 E	29-04-2002