

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
7 December 2006 (07.12.2006)

PCT

(10) International Publication Number  
**WO 2006/130090 A1**

(51) International Patent Classification:

A61K 38/45 (2006.01) A61K 31/437 (2006.01)  
A61K 31/275 (2006.01) A61P 9/10 (2006.01)  
A61K 31/404 (2006.01) G01N 33/68 (2006.01)

(21) International Application Number:

PCT/SE2006/000661

(22) International Filing Date: 2 June 2006 (02.06.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0501275-2 3 June 2005 (03.06.2005) SE

(71) Applicant (for all designated States except US): **PRONAS PHARMA AB** [SE/SE]; C/O Teknopol AB, Ideon, S-223 70 Lund (SE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **EDVINSSON, Lars** [SE/SE]; St Laurentilgatan 14 A, S-222 21 Lund (SE). **BEG, Saema** [SE/SE]; Månskäran 3, S-245 38 Staffanstorps (SE).

(74) Agent: **STRÖM & GULLIKSSON AB**; P.O. Box 4188, S-203 13 Malmö (SE).

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

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: USE OF A SUBSTANCE WHICH INHIBITS ENDOTHELIN TYPE B AND 5-HYDROXYTRYPTAMINE TYPE 1B RECEPTOR UPREGULATION IN THE TREATMENT OF ISCHEMIC DISORDERS

(57) Abstract: The invention relates to the use of at least one inhibitor, which prevents or reverts endothelin type B and 5-hydroxytryptamine type 1B receptor up regulation, for the manufacturing of a medicament for the treatment of an ischemic disorder or disease as well as a screening method to identify such an inhibitor.



WO 2006/130090 A1

Use of a substance which inhibits endothelin type B and 5-hydroxytryptamine type 1B receptor upregulation in the treatment of ischemic disorders.

## 5 FIELD OF INVENTION

The invention relates to the use of at least one inhibitor, which prevents or reverts endothelin type B and 5-hydroxytryptamine type 1B receptor upregulation, for the manufacturing of a medicament for the treatment of an ischemic disorder or disease, as well as a screening method to identify such an inhibitor.

10

## BACKGROUND OF THE INVENTION

Stroke is the third leading cause of death in many industrial countries. Around 20 % of the patients do not survive the first month and >30% who are alive 6 months later will be dependent on other people. Stroke is often ischemic and the majority of ischemic strokes are the result of an occlusion of a major cerebral artery by a thrombus or an embolism, which give rise to loss of blood flow in one or more specific regions.

Today there are two alternative ways for the treatment of stroke. One way is to try to establish reperfusion to the compromised region by dissolution of the clot using thrombolytic agents. Today, the recombinant tissue-plasminogen activator (rt-PA) is the only thrombolytic agent approved to be used for the treatment of acute ischemic brain injury. The use of rt-PA is restricted to administration within 3 hours after the stroke has occurred. However, its use increases the risk of haemorrhagic transformation, which limits its use. The second way is to develop compounds, which interfere with the biochemical pathway that leads to cell death. By such an approach the core area of injury will not be saved. However, the surrounding area called the ischemic penumbral area (or simply the penumbra) might be saved and the degree of damage restricted.

So far all the agents developed, which were supposed to save the penumbral area, failed to convincingly show efficacy in clinical trials despite they had shown good potential results in animal models. Examples of such compounds are NMDA receptor antagonists, Kappa opioid peptide receptor antagonists, NO inhibitors, Na<sup>+</sup> channel blockers, K<sup>+</sup> channel blockers, and cell membrane stabilisers, among others.

Other treatments are mainly focused on preventive pharmacotherapy, e.g., by use of antihypertensive agents, antilipids or anticoagulants. Current treatment also relates to alternate ways of cooling the patients, in order to mitigate the negative effect of a stroke. Therefore, recorded treatments are insufficient, or,

they can be considered as supportive and synergistic to future regimens emanating from the present invention.

The past 20 years have focused on the mechanisms of ischemic brain damage within the brain tissue per se; the work is related to free radical-mediated damage (Chan PH, Reactive oxygen radical signalling and damage in the ischemic brain. *J Cereb Blood Flow Metab* 2001;21:2-14 ), apoptosis ( MacManus JP, Buchan AM. Apoptosis after experimental stroke: fact or fashion? *J Neurotrauma* 2000;17:899-914 ), gene expression (Sharp FR et al. Multiple molecular penumbras after focal cerebral ischemia. *J Cereb Blood Flow Metab* 2000;20:1011-1032 ), and inflammation (Iadecola C, Alexander M. Cerebral ischemia and inflammation. *Curr Opin Neurol* 2001;14:89-94 ) all within the brain tissue/neurons (Wieloch T. Molecular mechanisms of ischemic brain damage. In *Cerebral Blood Flow and Metabolism*, ed by L Edvinsson and D N Krause, Lippincott Williams Wilkins, Philadelphia 2002, pp 423-451). In particular there has been a focus on NMDA receptors and calcium toxicity as a primary trigger in ischemia. This has been a target in pharmacology to search for the molecular mechanisms of ischemic brain damage and for neuroprotective compounds against ischemic injury. To date, however, no successful clinical stroke trial has appeared (Lees KR. Neuroprotection. *Br Med Bull* 2000;56:401-412 ). In all the work during the past 20 years focus has been directed towards the molecular mechanisms within the brain neurons. A hypothetical vascular involvement is regarded as a passive secondary event that by and large has been left out from the discussions.

Consequently, there is a need for agents to be used for the treatment of ischemic diseases or disorders, such as stroke as well as screening methods to enable the finding of new bioactive agents that can be used to efficiently treat ischemic brain injury, to save the penumbral area and help the patient to get a better quality of life. Today, such agents are not available, agents, which are safe, non-addictive and effective, and also to which the body in the long-term is not refractory.

30

## SUMMARY OF THE INVENTION

The invention relates to the finding that by inhibiting of endothelin type B and 5-hydroxytryptamine type 1B it is possible to prevent the cerebral blood flow (CBF) reduction associated with subarachnoid haemorrhage (SAH) in a model system of a rat. Previous approaches involving neuroprotection have proven promising for nerve cell survival in vitro, but these regimens have failed in clinical trials. An apparent reason for the lack of efficacy is the inability of neuroprotective drugs to reach their targets due to the compromised blood flow in the penumbra.

According to a first aspect the invention relates to the use of at least one inhibitor, which prevents or reverts endothelin type B and 5-hydroxytryptamine type 1B receptor up regulation for the manufacturing of a medicament for the treatment of an ischemic disorder or disease, such as stroke.

5 According to a second aspect the invention relates to a screening method to identify an inhibitor of endothelin type B and 5-hydroxytryptamine type 1B receptor up regulation comprising the steps of; providing dissected arteries from an animal, inducing at least the endothelin receptors, immersing said dissected arteries in a solution and applying a tension on said arteries, adding a bioactive  
10 agent to said immersed dissected arteries, determining the contractile response of said dissected arteries and obtaining a bioactive agent, which inhibits endothelin type B and 5-hydroxytryptamine type 1B receptor up regulation.

According to a third aspect the invention relates to the use of at least one biomarker to screen for an inhibitor of endothelin type B and 5-  
15 hydroxytryptamine type 1B receptor up regulation.

With the use of such inhibitors it is possible to treat a mammal in need thereof after an ischemic brain injury. By such a treatment there is an increased possibility that patients suffering from ischemic brain injury will recover and, subsequently, have better chances of a normal life, as compared to the treatments  
20 that are available today. According to a second embodiment the invention relates to a method to identify a bioactive agent obtainable by the disclosed method. The bioactive agent may be used alone or in combination with the therapeutic agents commonly used today and mentioned above for the treatment of ischemic brain injury.

25

## DETAILED DESCRIPTION OF THE INVENTION

### Definitions

In the context of the present application and invention the following definitions apply:

30 The term “an endothelin type B and 5-hydroxytryptamine type 1B inhibitor” is intended to mean any natural, synthetic or semisynthetic compound that may act as an inhibitor of the altered expression of endothelin type B and 5-hydroxytryptamine type 1B receptors and prevent reduction of CBF. The inhibitor may for example be a protein kinase inhibitor or part of a protein kinase inhibitor or  
35 a fusion of a protein kinase inhibitor and another component or part as long as it has the capability to inhibit endothelin type B and 5-hydroxytryptamine type 1B receptor upregulation and hence the function.

The term “ischemic brain injury” is intended to mean any kind of disorder or disease involving any ischemic event. One example is stroke.

The term "bioactive agent" is intended to mean an agent being capable of acting as an endothelin type B and 5-hydroxytryptamine type 1B inhibitor, such as being a protein kinase inhibitor or a MAPK inhibitor. The inhibitor may for example inhibit ERK1/2, *raf* or protein kinase alpha or delta and being capable of  
5 normalising vascular tone by decreasing vasoconstriction or increasing vasodilatation of blood vessels such as arteries after ischemic injury, such as ischemic brain injury, angina pectoris or ischemic heart disease.

The term "physiological solution" is intended to mean a solution, in which receptors of dissected arteries may be upregulating. Examples of receptors  
10 are endothelin, such as A<sub>1</sub> and A<sub>2</sub>, angiotensin, such as AT<sub>1</sub> and AT<sub>2</sub>, serotonin, such as 5-HT<sub>1B</sub> and bradykinin, such as B<sub>1</sub> and B<sub>2</sub>. Examples of physiological solutions will be obvious for a person skilled in the art. Examples are modified KREB's, DMEM, physiological NaCl, phosphate buffer, etc.

The term "treatment" according to this invention means that by  
15 inhibiting the signal resulting in transcription and translation, the receptor upregulation will be prevented and, hence, the sequelae leading to penumbral zone damage and neuronal loss will be revoked.

The term "screening method" according to our invention means that the applicability of said method to inhibit upregulation of receptors can be shown, e.g.,  
20 by using certain commercially available PK inhibitors and related compounds (for reference see, e.g., Hansen-Schwartz et al., Br J Pharmacol, 2002;137(1):118-126; Uddman et al., Eur J Pharmacol, 2002; 452(2):215-22).

#### Endothelin type B and 5-hydroxytryptamine type 1B upregulation inhibitor

25 The present invention relates to the area of treatment of vasoconstriction and/or vasodilation of arteries, such as human or animal arteries, including cerebral arteries. The idea being based on the hypothesis that down regulation of the endothelin type B (ET<sub>B</sub>) as well as the 5-hydroxytryptamine type 1B (5-HT<sub>1B</sub>) receptors prevent the cerebral blood flow (CBF) reduction associated  
30 with subarachnoid haemorrhage (SAH) or stroke after occlusion of a cerebral artery. The cerebral ischemia (induced by either SAH or artery occlusion) results in a change in the receptor expression in cerebral arteries involved. The receptor upregulation causes further contraction, which limits the oxygen delivery to the ischemic artery and thus worsens the area at risk; in SAH a second ischemia and in occlusion the  
35 development of the penumbra. In a series of experiments we have defined the signal transduction mechanisms leading to the key mechanism, the receptor upregulation. By inhibiting the process by e.g., *raf*, ERK1/2 or a PKC inhibitor we can not only reverse or counteract the vascular receptor upregulation but also reduce and prevent

the neuronal death and cerebral blood flow reduction. Thus by using this mechanism we can limit the damage in man after a stroke.

The invention relates to the use of an inhibitor, which prevents or reverts endothelin type B and 5-hydroxytryptamine type 1B receptor up regulation  
5 for the manufacturing of a medicament for the treatment of an ischemic disorder or disease. Examples of disorders or diseases are ischemic brain injury, such as stroke, angina pectoris, claudication and ischemic heart disease or disorder.

The inhibitors may be a protein kinase C, MAPKKK, MAPKK or MAPK inhibitor. The inhibitors may for example inhibit *raf*, MEK1/2 or  
10 extracellular signal-regulated kinase (ERK1/2). Examples of inhibitors are SB 386023, which inhibits *raf* and U0126, which inhibits ERK 1/2.

Alternatively the inhibitor is a protein kinase C inhibitor, such as a PKC alpha or delta inhibitor. Examples of inhibitors are RO-31-8220, RO-31-7549 or RO-32-0432.

15 Accordingly, a mixture of one or more inhibitors selected from the group consisting of protein kinase C, MAPKKK, MAPKK and MAPK may be used for the treatment of a ischemic disorder or disease.

Alternatively, the inhibitor is used for the inhibition of at least one transcription factor activity, wherein said transcription factor is selected from the  
20 group consisting of Elk-1, ATF-2 and c-Jun. These transcription factors become activated by p38, ERK 1/2 and SAPK/JNK and increase the expression of inflammatory and extracellular-matrix-related genes.

The invented medicament may further comprise a pharmaceutically acceptable diluent, excipient, buffer or carrier.

25 "Pharmaceutically acceptable" means a non-toxic material that does not decrease the effectiveness of the biological activity of the active ingredients. Such pharmaceutically acceptable buffers, carriers or excipients are well-known in the art (see Remington's Pharmaceutical Sciences, 18th edition, A.R Gennaro, Ed., Mack Publishing Company (1990) and handbook of Pharmaceutical Excipients, 3rd  
30 edition, A. Kibbe, Ed., Pharmaceutical Press (2000). The invented medicament may be placed in a container, such as an ampoule, syringe or the like.

To evaluate the hypothesis a subset of different inhibitors were evaluated (see the examples). First was a PKC inhibitor RO-31-7549 injected intracisternally in conjunction and induced SAH in rats. Two days following the  
35 SAH cerebral arteries were harvested. Only the PKC $\delta$  subtype was increased after SAH and this was not seen in RO-31-7549 treated SAH animals. The contractile responses to endothelin-1 (ET-1; ET<sub>A</sub> and ET<sub>B</sub> receptor agonist) and 5-carboxamidotryptamine (5-CT; 5-HT<sub>1</sub> receptor agonist) were increased after SAH. In parallel the ET<sub>B</sub> and 5-HT<sub>1B</sub> receptor mRNA and protein levels were elevated as

analyzed by quantitative real-time PCR and immunohistochemistry. Administration of RO-31-7549 prevented the enhanced contraction elicited by application of ET-1 and 5-CT in cerebral arteries compared to SAH and kept the ET<sub>B</sub> and 5-HT<sub>1B</sub> receptor mRNA levels at pre SAH levels. In addition regional CBF was evaluated by an autoradiographic technique. SAH induced a powerful reduction in CBF by 60%. Thereby it was concluded that by the use of a protein kinase inhibitor such as a protein kinase C subtype  $\delta$  inhibitor, it was possible keep the levels of ET<sub>B</sub> as well as 5-HT<sub>1B</sub> to normal levels as well as prevent CBF reduction. A second protein kinase inhibitor was also evaluated, i.e., Ro-32-0432.

Secondly were a MAPK inhibitor evaluated, an inhibitor, which inhibits of ERK1/2. As an example was the MAPKKK inhibitor SB386023(-b) used. It was injected intracisternally in conjunction with and after the induced SAH. Two days after the SAH cerebral arteries were harvested for quantitative real-time PCR, immunohistochemistry and the contractile responses to endothelin-1 (ET-1; ET<sub>A</sub> and ET<sub>B</sub> receptor agonist) and 5-carboxamidotryptamine (5-CT; 5-HT<sub>1</sub> receptor agonist) were carried out in a sensitive myograph. To investigate if MAPK inhibition had an influence on the local and global CBF after SAH an autoradiographic technique was used. The SAH reduced global and regional CBF by about 60% and this was prevented by treatment with SB386023-b. The ERK1/2 inhibition decreased the maximum contraction elicited by application of ET-1 and 5-CT in cerebral arteries compared to SAH. In parallel the ERK1/2 inhibition down regulated ET<sub>B</sub> and 5-HT<sub>1B</sub> receptor mRNA and protein levels compared to the SAH. Cerebral vasospasm after SAH involves vasoconstriction and subsequent reduction in the CBF. A second inhibitor being involved in the ERK pathways was also evaluated, i.e., U0126, which inhibits MEK1/2.

By the two experiments it was shown that by inhibiting the endothelin type B (ET<sub>B</sub>) as well as the 5-hydroxytryptamine type 1B (5-HT<sub>1B</sub>) receptors it is possible to prevent the reduction of CBF and thereby also possible to use these types of inhibitors for the treatment of disorders, such as stroke. This is a new principle and it may have a strong clinical value.

The inhibitor may be any inhibitor which can act as a MAPK inhibitor or a protein kinase inhibitor, such as being synthetic or semisynthetic or part thereof. The inhibitor may also be small peptides or a small chemical compound as long as it can act as an inhibitor as mentioned above.

#### A screening method according to the invention

The invention relates to a screening method to identify an endothelin type B and 5-hydroxytryptamine type 1B inhibitor, such as by the use of one or more biomarker as shown under the examples.

To be able to identify such bioactive agents there is a need for one or more model system in which vasoconstriction and/or vasodilatation may be induced in parallel or in sequence with upregulation of one or more receptors, such as endothelin, angiotensin, serotonin and/or bradykinin. The inventors have

5 surprisingly found that blood vessels of tissue subjected to ischemia, such as the basilar artery and the middle cerebral artery, undergoes phenotypic changes so that the vasoconstrictor receptor population is upregulated, which result in excessive arterial constriction, and that for example the endothelin and the 5-carboxamidotryptamine receptors are among those becoming upregulated.

10 Vasoconstriction and/or vasodilatation can be induced in a mammalian animal such as rat, mouse, guinea-pig, dog, cat, horse or a synthetic version thereof. For example by the intraluminal occlusion technique, originally described by Longa et al., Stroke, 1989; 20(1):84-91. After the animals have recovered from the surgery, and vasoconstriction and/or vasodilatation been induced to the tissue or organ, said  
15 specimen is removed from the animal for further analysis (see Example 1 and 2). Arteries from the tissue or organ may thus be isolated to be used within the disclosed method to identify new bioactive agents.

Another example is the use of isolated dissected arteries from a mammalian animal such as rat, mouse, guinea-pig, dog, cat, horse or a synthetic  
20 version thereof (see Example 3 and 4). The isolated arteries are exposed to a physiological solution, in which the endothelial receptors can be upregulated. Example of such a physiological solution is the nutrition solution Dulbecco's modified Eagle's medium (DMEM).

The arteries may be dissected into smaller segments or discs either prior  
25 to or after upregulation of one or more receptors, such as the endothelin receptors.

The tissue, organ, cells or arteries have the same characteristics as for example a patient who has suffered an ischemic brain injury.

The arteries with upregulated receptors will then be immersed in a solution to maintain their biological activities, also exposing the preparations to a  
30 tension, in order to enable the possibility to study vasoconstriction and/or vasodilatation. One way to study isometric tension is to use a Mulvany-Halpern Myograph (Danish Myo Technology A/S, Denmark). The arteries are then exposed to a bioactive agent and the contractile response studied. If the response is reduced the bioactive agent is determined as being a candidate able of acting as a kinas  
35 inhibitor, which may be used for the treatment of disorders involving vasoconstrict

The examples below are included to further illustrate the invention and the invention should not be limited thereto.

## EXAMPLE 1

## Cerebral Ischemia in vivo

5 Middle cerebral artery occlusion(MCA)

Focal cerebral ischemia was induced by the intraluminal occlusion technique originally described by Longa et al., 1989. Male Wistar Hannover rats (Møllegaard Breeding Center, Copenhagen, Denmark), weighing 350-420 g were used for the study. The experimental procedures used have been approved by the  
10 Ethics Committee for use of Laboratory Animal Experiments at the University of Lund (M217-00). The animals were fasted overnight with free access to water. Anaesthesia was induced with 4.5% halothane in N<sub>2</sub>O: O<sub>2</sub> (70: 30). They were kept anaesthetized on a mask, inhaling 1-1.5% halothane in N<sub>2</sub>O: O<sub>2</sub> (70: 30) during the surgical procedure. The right MCA was occluded by inserting a thin filament into  
15 the internal carotid artery and advance this to the origin of the MCA (further details are described by Stenman et al., Stroke, 2002; 33(9):2311-16). When the surgical procedures were finished, anaesthesia was discontinued and the animals were allowed to wake up. Two hours after the MCA occlusion, the animals were briefly re-anaesthetized to allow withdrawal of the filament to achieve reperfusion. Sham-  
20 operated rats went through the same operative procedure as the MCA-occluded rats, except that the filament was immediately withdrawn after insertion. These animals were re-anaesthetized for 15 min after two hours.

Removal of MCA and evaluation of ischemic damage in the rat

25 After 46 hours of recovery, the animals were re-anaesthetized and decapitated. The brains were quickly removed and chilled in ice-cold bicarbonate buffer solution (for composition, see below). The right and left MCA were removed, cut into segments and immediately denuded from endothelium before mounting in myographs or snap frozen at -80°C for examination by real-time PCR. The ischemic  
30 damage was confirmed in all animals by staining coronal slices of the brains with 1% 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in saline solution at 37°C for 20 min.<sup>8</sup>

## EXAMPLE 2

Rat subarachnoid haemorrhage (SAH) model

Subarachnoid haemorrhage was induced by a model originally devised  
5 by Svendgaard (Prunell et al., 2002) and carefully described by Prunell (Prunell et al., 2003).

Male Sprague-Dawley rats (350-400 g) were anaesthetized using 5%  
halothane (Halocarbon Laboratories, River Edge, New Jersey) in N<sub>2</sub>O/O<sub>2</sub> (30:70).  
The rat was intubated and artificially ventilated with inhalation of 0.5-1.5%  
10 halothane in N<sub>2</sub>O/O<sub>2</sub> (70:30) during the surgical procedure. The depth of anaesthesia  
was carefully monitored and the respiration checked by regularly withdrawing  
arterial blood samples for blood gas analysis (Radiometer, Copenhagen, Denmark).  
An arterial catheter to measure blood pressure was placed in the tail artery and a  
catheter to monitor intracranial pressure (ICP) was placed in the suboccipital  
15 membrane. At either side of the skull, 3 mm from the midline and 4 mm anteriorly  
from the bregma, holes were drilled through the skull bone down to dura mater  
(without perforation) allowing the placement of two laser-Doppler flow probes to  
measure CBF. Finally, a 27G blunt canula with side hole was introduced  
stereotactically 6.5 mm anterior to bregma in the midline at an angle of 30° to the  
20 vertical. With the aperture pointing to the right, the needle was lowered until the tip  
reached the skull base 2 to 3 mm anterior to chiasma, approximately 10 mm from  
the brain surface. After 30 minutes of equilibration 250 µl blood was withdrawn  
from the tail catheter and injected intracranially at a pressure equal to the mean  
arterial blood pressure (MABP) (80-100 mmHg). Subsequently the rat was kept  
25 under anaesthesia for another 60 minutes to allow recovery from the cerebral insult  
after which catheters were removed and the incisions closed. The rat was then  
revitalized and extubated. A subcutaneous injection of carprofen (4.0 mg/kg)  
(Pfizer, Denmark) was administered and the rat was hydrated subcutaneously using  
40 ml isotonic sodium chloride at the end of the operation and at day one. During  
30 the period of observation the rat was monitored regularly, and if showing severe  
distress the animal was prematurely killed. In addition, a series of sham-operated  
rats were prepared. They went through exactly the same procedure as described  
above with the exception that no blood was injected intracisternally. After two days  
either harvesting of vessels or autoradiographic measurements were done (see below  
35 for details).

Rat subarachnoid haemorrhage model with PKC inhibition

This group of animals went through the same procedure as the above-  
mentioned SAH animals. In addition they were treated with the PKC inhibitor RO-

31-7549 (Calbiochem, Sweden) in conjunction with the operation and after the induced SAH. RO-31-7549 (50  $\mu$ L;  $10^{-6}$  M) was injected intracisternally at 30 minutes prior to the induced SAH and after the SAH (20  $\mu$ L;  $10^{-6}$  M) RO-31-7549 was given at the time points 3, 6, 24 and 32 hour after the first RO-31-7549 injection. RO-31-7549 is a selective PKC inhibitor with some isozyme specificity, primarily inhibiting classical PKC's ( $IC_{50}$  for  $PKC_{\alpha}=10^{-7}$  M,  $PKC_{\beta I}=10^{-6.7}$  M,  $PKC_{\beta II}=10^{-6.8}$  M,  $PKC_{\gamma}=10^{-6.7}$  M and  $PKC_{\epsilon}=10^{-6.8}$  M) (Wilkinson et al., 1993).

#### Rat subarachnoid haemorrhage model with MAPK ERK1/2 inhibition

This group of animals went through the same procedure as the above-mentioned SAH animals. In addition they were treated with SB386023-b (a kind gift from Dr A A Parsons, GSK, UK) in conjunction with the operation and after the induced SAH. SB386023-b (50  $\mu$ L;  $10^{-6}$  M) was injected intracisternally at 30 minutes prior to the induced SAH and after the SAH (20  $\mu$ L;  $10^{-6}$  M) SB386023-b was given at time points 3, 6, 24 and 32 hours after the first SB386023-b injection.

#### Harvest of cerebral arteries

After 48 hours of observation the sham, SAH and SAH treated with RO-31-7549 operated rats (see above SAH model) were anaesthetized with  $CO_2$  and decapitated. The brains were quickly removed and chilled in ice-cold bicarbonate buffer solution (see composition below). Under a dissection microscope the middle cerebral artery (MCA), basilar artery (BA) and the circle of Willis were carefully dissected free from the brain and cleared of connective tissue. The MCA and BA were immediately mounted in myographs for in vitro pharmacology or snap frozen at  $-80^{\circ}C$  and examined by real-time PCR or immunohistochemistry. Circle of Willis arteries were directly snap frozen at  $-80^{\circ}C$  and used for Western blot investigation.

### EXAMPLE 3

#### Pharmacology in vitro

A distal branch of the rat superior mesenteric artery (outer diameter  $< 1$  mm) or basilar arteries (outer diameter 0.4-0.5 mm) was chosen as experimental target since previous studies have shown that endothelin  $ET_B$  receptors are up-regulated in this vessel following 24 h of incubation with Dulbecco's modified Eagle's medium (DMEM). Male Wistar-Kyoto rats (200-350g, M & B, Denmark) were anaesthetized with  $CO_2$  and killed by decapitation. The artery was removed, dissected free from adherent tissue and cut into 1 mm long circular segments. Segments were placed in a well containing 1 ml of DMEM, supplemented with

penicillin (100 U/ml) and streptomycin (100 mg/ml), and incubated at 37° C in humidified 5% CO<sub>2</sub> in air (pH 7.4).

Functional studies were performed using a sensitive *in vitro* system where ET-1 (endothelin ET<sub>A</sub> and ET<sub>B</sub> receptor agonist) and sarafotoxin 6c (S6c; selective ET<sub>B</sub> agonist) were used to induce endothelin receptor mediated contractile responses. To study the effect of different inhibitors on the ET receptor up-regulation, paired segments were incubated with inhibitors for 24 h before they were studied. The inhibitors were present in the medium during the incubation period only, not during the vasomotor tests.

After incubation the segments were immersed in temperature-controlled (37° C) tissue baths containing a bicarbonate buffer solution (for composition, see below). The solution was continuously aerated with 5% CO<sub>2</sub> in O<sub>2</sub> resulting in a pH of 7.4. Each segment was mounted on two L-shaped prongs, one of which was attached to a Grass FT-03 transducer (Grass Instr., Quincy, USA) connected to a MacLab unit (ADInstruments, Hastings, UK) for continuous recording of isometric tension. A tension of 2 mN was applied to each segment and the segments were allowed to stabilise at this tension for one hour before being exposed to a K<sup>+</sup>-rich (63,5 mM) buffer solution with the same composition as the standard solution except that NaCl was replaced by an equimolar concentration of KCl. The K<sup>+</sup>-induced contraction was used as a reference for the contractile capacity, and the segments was used only if K<sup>+</sup> elicited reproducible responses over 0.5 mN. Concentration-response curves for the specific endothelin ET<sub>B</sub> receptor agonist S6c were then obtained by cumulative administration of the peptide. The vessels were then washed and allowed to recover for one hour before ET-1 concentration-response curves were performed (for further methodological details see Uddman et al., 2002).

#### Buffer solutions and drugs

Standard buffer solution (mM): NaCl 119, NaHCO<sub>3</sub> 15, KCl 4.6, MgCl<sub>2</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 1.5, glucose 5.5. Analytical grade chemicals and double-distilled water were used for preparing all solutions. S6c and ET-1 (Auspeptide, Aus) were dissolved in water with bovine serum albumin (Kabi, Sweden) (0.1% w/v). All MAPK inhibitors were dissolved in DMSO and diluted in water. PD98059 was purchased from Sigma, St. Louis, USA. SB239063, SB408039, SB386023b and SB203580 were a generous gift from Dr A.A. Parsons, GSK, UK.

### Calculations and statistics

Data are expressed as mean values  $\pm$  S.E.M. Contractile responses in each segment are expressed as a percentage of the  $K^+$ -induced contraction.  $E_{max}$  represents the maximal contraction induced by an agonist, and given either in absolute values or expressed as a percentage of the  $K^+$ -induced response for each vessel segment. The  $pEC_{50}$  value was calculated from the line between the concentrations above and below the midpoint of the concentration-response curve. Kruskal-Wallis nonparametric test with Dunn's post-hoc test was used for all statistical analyses. Differences were considered significant at  $P < 0.05$ .

### EXAMPLE 4

#### Myograph in vitro

The vessels (MCA) were cut into cylindrical segments and the endothelium was removed mechanically by inserting a thin thread and rubbing the endothelium. The vessel segments were threaded on two 40  $\mu$ m diameter stainless steel wires and mounted in a Mulvany-Halpern myograph (Danish Myo Technology A/S, Denmark) for recording of isometric tension. One wire was connected to a force displacement-transducer attached to an analogue-digital converter unit (ADInstruments, Hastings UK). The other wire was attached to a movable displacement device allowing fine adjustments of vascular tension by varying the distance between the wires. The experiments were recorded on a computer by use of the software program Chart<sup>TM</sup> (ADInstruments, UK). The segments were immersed in temperature-controlled (37°C) tissue baths containing a bicarbonate buffer solution of the following composition (mmol/L): NaCl 119;  $NaHCO_3$  15; KCl 4.6;  $MgCl_2$  1.2;  $NaH_2PO_4$  1.2;  $CaCl_2$  1.5 and glucose 5.5. The solution was continuously gassed with 5%  $CO_2$  in  $O_2$ , resulting in a pH of 7.4. The vessels were given an initial tension of 1.2 mN, and were adjusted to this level of tension for 1 hour. The contractile capacity was determined by exposure to a potassium-rich (63.5 mmol/L) buffer solution with the same composition as the bicarbonate buffer solution except that NaCl was exchanged for KCl. The contraction to 63.5 mmol/L potassium was used as a reference for the contractile capacity. Removal of the endothelium was confirmed by monitoring responses to  $10^{-5}$  M acetylcholine (ACh) after precontraction with 5-hydroxytryptamine (5-HT).

Concentration-response curves for Ang II, 5-HT, 5-CT, endothelin and sarafotoxin 6c ( $10^{-12}$ - $10^{-6.5}$  mol/L) were obtained by cumulative application. In order to characterize the receptor type responsible for the responses, two different specific receptor antagonists: candesartan ( $10^{-7}$  mol/L), losartan ( $10^{-7}$  mol/L), and  $AT_2$  receptor antagonist, PD123319 ( $10^{-6}$  mol/L) for angiotensin receptors;

bosentan, FR139317 and BQ123 for endothelin receptors; ketanserin, BRL or xyz for 5-HT receptors; were added to the buffer solution 30 minutes before application of either agonist (in separate experiments).

## 5 Calculations

*Pharmacology:* Contractile experiments were performed with 5-8 rats per group (1-2 segments from each vessel). For experiments with Ang II receptor blockers, 3 occluded MCAs were treated with losartan and PD123319, and 2 occluded MCAs were treated with candesartan. The  $E_{\max}$  values refer to maximum contraction calculated as percentage of the contractile capacity of 63.5 mM  $K^+$ . Data are expressed as mean values  $\pm$  standard error of the mean (SEM). Statistical analyses were performed with one-way ANOVA followed by Dunnett's Multiple Comparison test.  $P < 0.05$  was considered significant.

## 15 EXAMPLE 5

### PCR Real-time

Segments to be used for real-time PCR were cultured, or removed from stroke or SAH rats, snap frozen in liquid nitrogen and subsequently stored at  $-80^{\circ}\text{C}$  until use. The total cellular RNA was extracted using the FastRNA, Pro Green kit (Qbiogene) for 60 seconds in the FastPrep FP120 instrument (Qbiogene) following the suppliers' instructions. The resulting pellet was finally washed with 70% ethanol, air-dried and redissolved in 50  $\mu\text{L}$  diethyl-pyrocabonate (DEPC)-treated water. Reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA PCR kit (PE Applied Biosystems, Foster City, CA, USA) in a Perkin-Elmer DNA Thermal cycler. First strand cDNA was synthesized from 1  $\mu\text{g}$  total RNA in a 20  $\mu\text{L}$  reaction volume using random hexamers as primers. The reaction mixture was incubated at  $25^{\circ}\text{C}$  for 10 min,  $48^{\circ}\text{C}$  for 15 min, heated to  $95^{\circ}\text{C}$  for 5 min and chilled to  $5^{\circ}\text{C}$  for 5 min. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System using the GeneAmp SYBR<sup>®</sup> Green kit (Perkin-Elmer, Applied Biosystems Foster City, CA, USA) with the cDNA synthesised above as template in a 50  $\mu\text{L}$  reaction volume. A no template control was included in all experiments. The GeneAmp 5700 Sequence Detection System monitors the growth of DNA in real-time using an optics- and imaging system, via the binding of a fluorescent dye to double-stranded DNA. Specific primers were designed as follows:

|                          |          |                             |
|--------------------------|----------|-----------------------------|
| ET <sub>A</sub> receptor | forward; | 5'-GTCGAGAGGTGGCAAAGACC -3' |
|                          | reverse; | 5'-ACAGGGCGAAGATGACAACC -3' |

ET<sub>B</sub> receptor                      forward;    5'-GATACGACAACTTCCGCTCCA-3'  
                                          reverse;    5'-GTCCACGATGAGGACAATGAG-3'

5                      Elongation factor-1 (EF-1) mRNA was used as a reference, since it is the product of a housekeeping gene, continuously expressed to a constant amount in cells. The EF-1 primers were designed as follows:

EF-1                      forward;    5'-GCAAGCCCATGTGTGTTGAA-3'  
 10                                           reverse;    5'-TGATGACACCCACAGCAACTG-3'

The real-time PCR was carried out with the following profile: 50° C for 2 min, 95° C for 10 min, followed by forty cycles with 95° C for 15 sec and 60° C for 1 min. To prove that the cDNA of EF-1 and the ET receptors were amplified  
 15 with the same efficacy during real-time PCR, standard curves were made where the C<sub>T</sub>-values were plotted against cDNA concentration based on the equation:

$$C_T = \lg \left( \frac{\text{concentration}}{(1 + E)} \right)$$

20                      where E is the amplification efficiency with the optimal value of 1. The amplification efficiency can be calculated from the slope of the standard curve with an ideal slope close to 3.3 (Fig. 1).

The amount of endothelin ET<sub>B</sub> receptor mRNA was calculated as relative to the amount of EF-1 mRNA in the same sample by the formula:

25                      
$$X_0 / R_0 = 2^{(C_{tR} - C_{tX})}$$

30                      where X<sub>0</sub> = original amount of ET receptor mRNA, R<sub>0</sub> = original amount of EF-1 mRNA, C<sub>tR</sub> = C<sub>T</sub>-value for EF-1 and C<sub>tX</sub> = C<sub>T</sub>-value for the ET receptor. Statistical analyses were performed with Kruskal-Wallis nonparametric test with Dunn's post-hoc test, where P < 0.05 was considered significant.

To verify that each primer-pair only generated one PCR product at the expected size, the real-time PCR products were separated electrophoretically in a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and subsequently viewed  
 35 in a UV-box. The expected sizes of primers were: ET<sub>A</sub> 64 base pairs and ET<sub>B</sub> 86 base pairs.

## EXAMPLE 6

Molecular biology of other receptors

Data suggest that other receptors. Examples of physiological solutions will AT<sub>1</sub> 5HT<sub>1B</sub> and B<sub>1</sub>, respectively, also are upregulated. There might be still other not yet defined receptors that are changed or over expressed in situations of stroke, which also could contribute to the excessive vasoconstriction in the penumbra. Incidentally, not only are membrane bound receptors contributing to regulation of blood flow, as their coupling with the activity of different types of ion channels are also relevant. Taken together, an effective method to identify potentially beneficial bioactive agents would require the ability to keep track of different vasomotor mechanisms.

*Extraction of total RNA:* Total cellular RNA was extracted using the FastRNA™ Kit-GREEN (BIO 101, Carlsbad CA, USA) following the suppliers' instructions. The resulting pellet was finally washed with 75% ethanol, air-dried and redissolved in 40 µL diethyl-pyrocabonate (DEPC)-treated water.

Reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA PCR kit (PE Applied Biosystems, Foster City, CA, USA) in a Perkin-Elmer DNA Thermal cycler. First strand cDNA was synthesized from total RNA in a 40 µL reaction volume using random hexamers as primers. The reaction mixture was incubated at 25°C for 10 min, heated to 42°C for 15 min, further heated to 99°C for 5 min and chilled to 5°C for 5 min. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System using the GeneAmp SYBR® Green kit (Perkin-Elmer, Applied Biosystems Foster City, CA, USA) with the cDNA synthesized above as template in a 50 µL reaction volume. A no template control was included in all experiments. The GeneAmp 5700 Sequence Detection System monitored the growth of DNA in real-time using an optic and imaging system, via the binding of a fluorescent dye to double-stranded DNA. Specific primers for the AT<sub>1</sub> and AT<sub>2</sub> receptors, ACE and NF-κB were designed as follows:

30

AT<sub>1</sub> receptor

forward: 5'-

GGATGGTTCTCAGAGAGAGTACAT-3'

reverse: 5'-CCTGCCCTCTTGTACCTGTTG

-3'

35 AT<sub>2</sub> receptor

forward: 5'-

TCTGTTAGTGGGATGCATGTAATCA-3'

reverse: 5'-TGTGGGCCTCCAAACCATT-3'

ACE

forward: 5'-

CCCGGAAATACGAAGAATTGC-3'

reverse: 5'-GGCTCTCCCCACCTTGTCTC-  
 3'  
 NF-κB forward: 5'-  
 GAGAGCCAGTAGCACGCATG-3'  
 5 reverse: 5'-CCTGGGTTCGTGGAATGAGT-  
 3'

Elongation factor-1 (EF-1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were used as endogenous standards, and the  
 10 primers were designed as follows:

EF-1 forward: 5'-  
 GCAAGCCCATGTGTGTTGAA-3'  
 reverse: 5'-  
 15 TGATGACACCCACAGCAACTG-3'  
 GAPDH forward: 5'-GGCCTTCCGTGTTCTACC-3'  
 reverse: 5'-CGGCATGTCAGATCCACAAC-  
 3'

20 The real-time PCR was carried out with the following profile: 50°C for 2 min, followed by 40 cycles with 95°C for 15 sec and 60°C for 1 min.

### Calculations

PCR: PCR experiments were performed on 5-6 MCA-occluded rats per  
 25 tested primer (each sample in duplicate). The amount of mRNA was calculated as relative to the amount of EF-1 and GAPDH mRNA in the same sample by the formula:  $X_0/R_0 = 2^{CtR-CtX}$ , where  $X_0$  = original amount of target mRNA,  $R_0$  = original amount of EF-1/GAPDH mRNA,  $CtR$  =  $C_T$ -value for EF-1/GAPDH and  $CtX$  =  $C_T$ -value for the target. By  $C_T$ -values we refer to the number of PCR cycles  
 30 performed per gene product in one sample at a specific point of time. Data are expressed as mean values  $\pm$  SEM. Statistical analyses were performed with unpaired Students' t-test and  $P < 0.05$  was considered significant.

### EXAMPLE 7

35

### Western blot

Arteries collected from the base of the brain (circle of Willis) were homogenized in lysis-buffer with protease- and phosphatase inhibitors (10 mM Tris pH 7.4, 50 mM  $\beta$ -Glycerophosphate, 100  $\mu$ M  $Na_3VO_4$ , 0.5% Deoxycholate, 1 mM

EGTA, 1 mM EDTA, 1 mM NaF, 20 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 1% Triton X-100, 1 mM DTT, 20  $\mu\text{M}$  Pepstatin, 20  $\mu\text{M}$  Leupeptin, 0.1 U/ml Aprotinin, 1 nM Calyculin and 1 mM PMSF). Total protein concentration was determined using a BioRad DC kit (Hercules, CA, USA) and measuring absorbance at 750 nm on a Genesys 10 spectrophotometer (Thermo, Waltham, MA, U.S.A.). 25  $\mu\text{g}$  total protein was loaded per lane on a 10% SDS-PAGE gel and blotted onto a Hybond PVDF membrane for 30 min at 1.5 mA/cm<sup>2</sup>. Subsequently the membrane was blocked in 5% non-fat milk for 1 hour at room temperature and incubated with primary antibody over-night at 4°C and with secondary antibody (Pierce, Rockford, IL, U.S.A.) for 1 hour at room temperature, with 3x5 min wash after antibody incubation. The membranes were developed using the Supersignal Dura kit (Pierce, Rockford, IL, U.S.A.) and visualized using a Fujifilm LAS-1000 Luminiscent Image Analyzer (Stamford, CT, U.S.A.). Antibodies against pPKC $\alpha_{\text{THR638}}$ , pPKC $\delta_{\text{SER645}}$ , pPKC $\epsilon_{\text{SER729}}$ , (Biosource, Camarillo, CA, U.S.A.), and ET<sub>A</sub> and ET<sub>B</sub> (Chemicon, Temecula, CA, U.S.A.) were used at 1:1000.

## EXAMPL 8

### Culture of vascular SMC

Male Sprague-Dawley rats (body weight 160g) were anesthetized by CO<sub>2</sub>, the animals were rapidly decapitated, the brain removed. The whole brain was gently homogenized in PBS with a glass homogenizer several times. Cerebral vessels were then isolated by 15% dextran density centrifugation at 3500 x g for 45 min. The cerebral vessels were collected from the bottom of the centrifugation tubes and incubated with collagenase/dispase (1 mg/ml) at 37°C for 1 h. The collagenase/dispase was removed by centrifugation 2000 x g for 10 min. The segments of cerebral vessel were then explanted into Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL). The DMEM was supplemented with 10% fetal bovine serum (FBS) (GibcoBRL, heated inactivated), bFGF 2 ng/ml, EGF 5 ng/ml, penicillin 100 U/ml and streptomycin 100 mg/ml. Subcultures were obtained by use of 0.25% trypsin-1mM EDTA (GibcoBRL).

Rat aortic and cerebral SMC were grown from explanted segments of the vessels. The endothelium was removed by scraping the intimal surface with a surgical blade. The medial layer of the vessel wall was removed and cut into 1x1 mm segments. The segments were grown in the same medium as mentioned above.

SMC were identified by more than 95% positive reaction of immunofluorescence staining with monoclonal antibody (mouse IgG) against alpha-smooth muscle actin and a secondary anti-mouse antibody labelled with FITC (Boehringer Mannheim, Germany) and in addition of a typical "hill and valley"

growth pattern. Cell viability was checked through all experiments by trypan blue (GibcoBRL) exclusion (>95%).

## EXAMPLE 9

5

### Autoradiographic measurements of regional CBF

Local cerebral blood flow was measured by a model originally described by Sakurada et al (Sakurada et al. 1978) and modified by Gjedde et al (Gjedde et al. 1980).

10 In brief, after 48 hours of observation rats in the various groups (sham, SAH and SAH treated with SB386023-b) were anaesthetized using 5% halothane in N<sub>2</sub>O/O<sub>2</sub> (30:70). The animal was intubated and artificially ventilated with inhalation of 0.5 -1.5% halothane in N<sub>2</sub>O/O<sub>2</sub> (70:30) during the surgical procedure. The anaesthesia and the respiration were monitored by regularly withdrawing arterial  
15 blood samples for blood gas analysis. A catheter to measure MABP was placed in the right femoral artery and a catheter for blood sampling was placed in the left femoral artery. This catheter was connected to a constant velocity withdrawal pump (Harvard apparatus 22, USA) for mechanical integration of tracer concentration. In addition, a catheter was inserted in one femoral vein for injection of heparin and for  
20 infusion of the radioactive tracer. The MABP was continuously monitored and a temperature probe was inserted into the rectum to record the temperature, which was regularly maintained at 37°C by a heating table. The haematocrit was measured by a haematocrit centrifuge (Beckman Microfuge 11, USA).

After 30 minutes of equilibration a bolus injection of 50 uCi of <sup>14</sup>C-  
25 iodoantipyrine 4[N-methyl-<sup>14</sup>C] (Perkin-Elmer, Boston, USA) was given i.v.. Arterial blood (122 µl) was withdrawn over 20 seconds. Immediately after this the animal was decapitated, the brain removed and immersed in isopentane (J.T. Baker, Deventer, Netherlands) chilled to - 50°C.

The arterial blood sample was transferred to liquid scintillation  
30 counting vials containing 1 ml mixture of Soluene-350 (Perkin-Elmer, Boston, USA) and Isopropanol (J.T. Baker, Deventer, Netherlands) (1:1). After 2 hours at 60°C 0.2 ml of 30% hydrogen peroxide was added to the vials, and the samples were maintained at room temperature for 15-30 minutes. Thereafter the samples were kept at 60°C for 30 minutes and 10-15 ml Hionic-Fluor (Perkin-Elmer, Foster, CA,  
35 USA) was added. The β-radioactivity scintillation counting was performed on the samples with a program that included quench correction ( Packard 2000 CA, Denmark). The <sup>14</sup>C activity in the tissue was determined after sectioning the brain in 20 µm sections at -20°C in a cryostat (Wild Leitz A/S, Glostrup, Denmark). The sections were exposed to x-ray films (Kodak, Denmark) together with <sup>14</sup>C

methacrylate standards (Amersham Life Science, England) which exposed the films for 20 days. Densities of the autoradiograms were measured with a Macintosh computer equipped with an analog CF 4/1 camera (Kaiser, Germany) and a transparency flat viewer (Color-Control 5000, Weilheim, Germany). The  $^{14}\text{C}$  content was determined in several brain regions (see Table 1). The CBF was calculated from the brain tissue  $^{14}\text{C}$  activity determined by autoradiography using Gjedde et al.'s equation (Gjedde et al. 1980):

$$f^{bl} = \frac{{}^{14}\text{C}_{Br}(T)}{E(T) \int_0^T C_a(t) dt}$$

#### EXAMPLE 10

In this example the aim was to examine the effect of inhibition of the MAP kinase/ERK kinase (MEK)1/2 on endothelin receptor alteration, brain damage and neurology in experimental cerebral ischaemia.

Transient middle cerebral artery occlusion was induced in male Wistar rats by the intraluminal filament technique as described above. The animals received 100 mg/kg i.p of the MEK1/2 inhibitor U0126 or vehicle in conjunction with the occlusion. After 24 hours the rats were decapitated and the brains removed. The middle cerebral arteries were dissected out and examined with myographs as described above or immunohistochemistry. The ischaemic areas of the brains were compared.

#### Immunohistochemistry

The MCAs were placed onto Tissue TEK (Gibco, Stockholm, Sweden), frozen and subsequently sectioned into 10  $\mu\text{m}$  slices. The primary antibodies used were rabbit anti-phospho-p44/42 MAPK (pERK1/2; Cellsignalling, Santa Cruz, USA) and rabbit anti-phospho-Elk-1 (Cellsignalling) diluted 1:50. The secondary antibody used was biotin conjugated goat anti-rabbit (JacksonImmunoResearch, Sohan, UK) diluted 1:100. All dilutions were done in PBS with 0.3% Triton-X100 and 5% foetal calf serum. The Vectastain ABC kit (Vector Laboratories, Burlingame, USA) together with DAB substrate (Rockland, Gilbertsville, USA) were used for detection according to the manufacturers' instructions. Pictures were taken at 40x magnification. As control, only secondary antibodies were used.

After the middle cerebral artery occlusion, the contractile responses of the endothelin A and B receptors were augmented in the ipsilateral middle cerebral artery. U0126 decreased this alteration in endothelin receptor response.

Furthermore, treatment with U0126 significantly decreased the brain damage and improved neurological scores. Immunohistochemistry showed that there were lower protein levels of the extracellular signal-regulated kinases (ERK)1/2 and the transcription factor Elk-1 in the U0126-treated rats compared to control. The results show that treatment with the MEK1/2 inhibitor U0126 in ischaemic stroke decreases brain damage, neurological symptoms and endothelin receptor alteration.

#### EXAMPLE 11

This example aimed to examine the effect of PKC inhibition on endothelin receptor upregulation, brain damage and neurology in experimental cerebral ischemia.

Transient middle cerebral artery occlusion was induced in male Wistar rats by the intraluminal filament technique as described above. The animals received 30 mg/kg i.p of the general PKC inhibitor Ro-32-0432 or vehicle in conjunction with the occlusion. After 24 hours the rats were sacrificed and the brains removed. The middle cerebral arteries were dissected out and their contractile properties examined with myographs as described above. The brains were stained with TTC for quantitative determination of ischemic damage.

24 hours after the middle cerebral artery occlusion, the contractile endothelin B receptor mediated response was upregulated in the ipsilateral middle cerebral artery of the control rats. In Ro-32-0432 treated rats, this upregulated endothelin receptor response was attenuated. Furthermore, Ro-32-0432 treatment decreased the brain damage significantly and improved neurological scores. The results suggest that treatment with Ro-32-0432 in ischemic stroke decreases the brain damage, neurological symptoms and associated ET<sub>B</sub> receptor upregulation.

#### EXAMPLE 12

##### *Immunohistochemistry*

The rat MCA was dissected out, treated as described above and placed in Tissue TEK (Gibco), frozen and subsequently sectioned into 10  $\mu$ m thick slices in a calibrated Microm HM500M cryostat (Microm, Germany). The primary antibodies used were rabbit antiphospho p38 (Cellsignalling #4631), rabbit antiphospho ERK 1/2 MAPK (Cellsignalling, #4376), rabbit antiphospho SAPK/JNK (Cellsignalling, #9251) rabbit antiphospho ATF-2 (Cellsignalling #9221), rabbit

antiphospho Elk-1 (Cellsignalling, #9181) and rabbit antiphospho c-Jun(Cellsignalling, #9261) each diluted 1:50. The secondary antibodies used were biotin-conjugated goat anti-rabbit antibodies (JackssonImmuno, 111-065-003) diluted 1:100. All dilutions were with PBS containing 0.3% Triton-X100 and 5% fetal calf serum. The Vectastain ABC kit (Vector Laboratories) together with DAB substrate (Rockland) were used for detection in accordance with the manufacturers' instructions. Pictures were taken at 40X magnification. Secondary antibodies alone served as a control. The results show that ATF-2, Elk-1 as well as c-Jun is activated.

## CLAIMS

1. Use of at least one inhibitor, which prevents or reverts endothelin type B and 5-hydroxytryptamine type 1B receptor up regulation for the manufacturing of  
5 a medicament for the treatment of an ischemic disorder or disease.
2. Use according to claim 1, wherein said disorder or disease is selected from the group consisting of ischemic brain injury, angina pectoris, claudication and ischemic heart disease or disorder.
3. Use according to any of preceding claims, wherein said inhibitor is selected  
10 from the group consisting of protein kinase C, MAPKKK, MAPKK and MAPK inhibitors.
4. Use according to claim 3, wherein said MAPKKK inhibitor inhibits *raf*.
5. Use according to claim 3, wherein said MAPKK inhibitor inhibits MEK1/2.
6. Use according to claim 4, wherein said MAPK inhibitor inhibits extracellular  
15 signal-regulated kinase (ERK1/2).
7. Use according to claim 6, wherein the protein kinase C inhibitor is a protein kinase C subtype alpha or delta inhibitor.
8. Use according to any of preceding claims, wherein a mixture of one or more inhibitors selected from the group consisting of protein kinase C, MAPKKK,  
20 MAPKK and MAPK is used.
9. Use of an inhibitor for the inhibition of at least one transcription factor activity, wherein said transcription factor is selected from the group consisting of Elk-1, ATF-2 and c-Jun.
10. Use according to any of preceding claims, wherein said medicament further  
25 comprises a pharmaceutically acceptable diluent, excipient, buffer or carrier.
11. A container comprises the medicament according to any of preceding claims.
12. A screening method to identify an inhibitor of endothelin type B and 5-hydroxytryptamine type 1B receptor up regulation comprising the steps of;
  - a. providing dissected arteries from an animal,
  - 30 b. inducing at least the endothelin receptors,
  - c. immersing said dissected arteries in a solution and applying a tension on said arteries,
  - d. adding a bioactive agent to said immersed dissected arteries,
  - e. determining the contractile response of said dissected arteries and
  - 35 f. obtaining a bioactive agent, which inhibits endothelin type B and 5-hydroxytryptamine type 1B receptor up regulation.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE2006/000661

## A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

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)

IPC: A61K, G01N

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

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, TXTE, MEDLINE, BIOSIS, EMBASE, CAPLUS, REGISTRY

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                         | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | HANSEN-SCHWARTZ J. "Receptor changes in cerebral arteries after subarachnoid haemorrhage", Acta Neurologica Scandinavica 2004, Vol. 109, No. 1, p. 33-44, page 40, left column, second paragraph, "Major conclusions", abstract<br>--                      | 1-12                  |
| X         | HANSEN-SCHWARTZ JACOB ET AL, "Protein kinase mediated upregulation of endothelin A, endothelin B and 5-hydroxytryptamine 1B/1D receptors during organ culture in rat basilar artery", British Journal of Pharmacology 2002, Vol. 137, p. 118-126, abstract | 11                    |
| A         | --                                                                                                                                                                                                                                                         | 1-10,12               |

☒ 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 application or patent 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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

31 August 2006

Date of mailing of the international search report

20-09-2006

Name and mailing address of the ISA/  
Swedish Patent Office  
Box 5055, S-102 42 STOCKHOLM  
Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Palmcrantz/ELY  
Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/ SE2006/000661**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.: 1-11  
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:  
  
Present claims 1-11 relate to an inhibitor defined by reference to a desirable characteristic or property, namely
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 fee, this Authority did not invite payment of any additional fee.
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.

## Box II.2

that the inhibitor should prevent or revert ETB and 5-HT1B receptor up-regulation, or that it is an inhibitor of PKC, MAPKKK, MAPKK, MAPK, raf, MEK1/2, ERK1/2 or an inhibitor of transcription factor activity. The intended inhibitors of claim 9 (of which there are no specific examples) is not even restricted to be used in the treatment of ischemic disorders. The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lacks clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to inhibitors of ETB and 5-HT1B receptors in general, such as inhibitors of PKC, MAPKKK, MAPKK, MAPK, raf, MEK1/2, ERK1/2 in general, and Elk-1, ATF-2 and c-Jun in general. The only compounds that have been searched specifically are RO-31-8220, RO-31-7549, RO-32-0432, U0126 and SB386023 (c.f. the description page 5, lines 8-15).

Further, claim 8 relates to the use of inhibitors selected from the group consisting of protein kinase C, MAPKKK, MAPKK and MAPK. It has been assumed that the intended inhibitors are selected from the group consisting of protein kinase C, MAPKKK, MAPKK and MAPK **inhibitors**, and the international search is based on this assumption.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE2006/000661

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                 | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | HENRIKSSON MARIE ET AL, "Importance of ERK1/2 in upregulation of endothelin type B receptors in cerebral arteries", British Journal of Pharmacology 2004, Vol. 142, p. 1155-1161, abstract                                                                                                                         | 11                    |
| A         | ---                                                                                                                                                                                                                                                                                                                | 1-10,12               |
| X         | EP 0365863 A2 (MERRELL DOW PHARMACEUTICALS INC.),<br>2 May 1990 (02.05.1990), page 2,<br>line 22 - line 24                                                                                                                                                                                                         | 11                    |
| X         | WO 0036150 A1 (ISIS PHARMACEUTICALS, INC.),<br>22 June 2000 (22.06.2000), the claims                                                                                                                                                                                                                               | 9                     |
| X         | WO 03068754 A1 (ASTRAZENECA AB), 21 August 2003<br>(21.08.2003), page 3, line 16 - line 18                                                                                                                                                                                                                         | 9                     |
| X         | SATOH MOTOYOSHI ET AL, "Inhibitory Effect With Antisense Mitogen-Activated Protein Kinase Oligodeoxynucleotide Against Cerebral Vasospasm in Rats", Stroke Journal of the American Heart Association 2002, Vol. 33, p. 775-781, Print ISSN 0039-2499. Online ISSN 1524-4628 page 780, left column, third paragraph | 1-3,10-11             |
| X         | NAMURA SHOBU ET AL, "Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia", PNAS September 25, 2001, Vol. 98, No. 20, p. 11569-11574, the abstract                                                                                    | 1-3,6,10-11           |
| X         | WO 2004064733 A2 (BOARD OF REGENTS, UNIVERSITY OF TEXAS SYSTEM), 5 August 2004 (05.08.2004), claim 1                                                                                                                                                                                                               | 1-4,10-11             |
|           | -----                                                                                                                                                                                                                                                                                                              |                       |

**International patent classification (IPC)**

**A61K 38/45** (2006.01)  
**A61K 31/275** (2006.01)  
**A61K 31/404** (2006.01)  
**A61K 31/437** (2006.01)  
**A61P 9/10** (2006.01)  
**G01N 33/68** (2006.01)

**Download your patent documents at [www.prv.se](http://www.prv.se)**

The cited patent documents can be downloaded at [www.prv.se](http://www.prv.se) by following the links:

- In English/Searches and advisory services/Cited documents (service in English) or
- e-tjänster/anförda dokument (service in Swedish).

Use the application number as username.

The password is **UMSQXMSNHJ**.

Paper copies can be ordered at a cost of 50 SEK per copy from PRV InterPat (telephone number 08-782 28 85).

Cited literature, if any, will be enclosed in paper form.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

04/03/2006

International application No.

PCT/SE2006/000661

|       |            |    |            |    |             |     |            |
|-------|------------|----|------------|----|-------------|-----|------------|
| EP    | 0365863    | A2 | 02/05/1990 | AR | 246733      | A   | 30/09/1994 |
|       |            |    |            | AT | 110074      | T   | 15/09/1994 |
|       |            |    |            | AU | 613302      | B   | 25/07/1991 |
|       |            |    |            | AU | 4249389     | A   | 26/04/1990 |
|       |            |    |            | CA | 2000804     | A,C | 24/04/1990 |
|       |            |    |            | CA | 2214533     | A,C | 24/04/1990 |
|       |            |    |            | CN | 1025854     | B,C | 07/09/1994 |
|       |            |    |            | CN | 1042145     | A   | 16/05/1990 |
|       |            |    |            | DE | 68917556    | D,T | 15/12/1994 |
|       |            |    |            | DK | 461389      | A   | 25/04/1990 |
|       |            |    |            | ES | 2061863     | T   | 16/12/1994 |
|       |            |    |            | FI | 93112       | B,C | 15/11/1994 |
|       |            |    |            | FI | 894459      | A   | 25/04/1990 |
|       |            |    |            | HU | 58088       | A   | 28/01/1992 |
|       |            |    |            | HU | 895410      | D   | 00/00/0000 |
|       |            |    |            | IE | 301189      | L   | 24/04/1990 |
|       |            |    |            | IE | 893011      | L   | 24/04/1990 |
|       |            |    |            | IL | 91788       | A   | 12/04/1994 |
|       |            |    |            | JP | 2164878     | A   | 25/06/1990 |
|       |            |    |            | JP | 2879454     | B   | 05/04/1999 |
|       |            |    |            | JP | 2983970     | B   | 29/11/1999 |
|       |            |    |            | JP | 11152285    | A   | 08/06/1999 |
|       |            |    |            | KR | 162637      | B   | 01/12/1998 |
|       |            |    |            | NO | 894210      | A   | 25/04/1990 |
|       |            |    |            | NZ | 230718      | A   | 27/11/1990 |
|       |            |    |            | PH | 27450       | A   | 02/07/1993 |
|       |            |    |            | PT | 91870       | A,B | 30/04/1990 |
|       |            |    |            | US | 4886811     | A   | 12/12/1989 |
|       |            |    |            | US | 4990519     | A   | 05/02/1991 |
|       |            |    |            | US | 4999431     | A   | 12/03/1991 |
|       |            |    |            | ZA | 8907259     | A   | 25/07/1990 |
| <hr/> |            |    |            |    |             |     |            |
| WO    | 0036150    | A1 | 22/06/2000 | AU | 4442799     | A   | 03/07/2000 |
|       |            |    |            | US | 5948680     | A   | 07/09/1999 |
| <hr/> |            |    |            |    |             |     |            |
| WO    | 03068754   | A1 | 21/08/2003 | AU | 2002239200  | A   | 00/00/0000 |
|       |            |    |            | AU | 2003206343  | A   | 00/00/0000 |
|       |            |    |            | EP | 1400094     | A   | 24/03/2004 |
|       |            |    |            | EP | 1476432     | A   | 17/11/2004 |
|       |            |    |            | JP | 2005519074  | T   | 30/06/2005 |
|       |            |    |            | SE | 0200450     | D   | 00/00/0000 |
|       |            |    |            | US | 20050113370 | A   | 26/05/2005 |
|       |            |    |            | US | 20050123127 | A   | 09/06/2005 |
|       |            |    |            | WO | 03077508    | A   | 18/09/2003 |
|       |            |    |            | SE | 0203122     | D   | 00/00/0000 |
| <hr/> |            |    |            |    |             |     |            |
| WO    | 2004064733 | A2 | 05/08/2004 | JP | 2004344879  | A   | 09/12/2004 |
|       |            |    |            | US | 6805433     | B   | 19/10/2004 |
|       |            |    |            | US | 20050250837 | A   | 10/11/2005 |