Title: USE OF 5-HT\textsubscript{1} RECEPTOR AGONISTS FOR THE TREATMENT OF PAIN

Abstract: The present invention refers to the use of 5-HT\textsubscript{1} receptor agonists for the treatment of pain and the symptoms of pain, especially certain subtypes of pain like neuropathic pain and inflammatory pain and symptoms involving allodynia and hyperalgesia, the prevention or the prophylaxis of pain and the symptoms of pain, especially certain subtypes of pain like neuropathic pain and inflammatory pain and symptoms involving allodynia and hyperalgesia.
Use of 5-HT₁ receptors agonists for the treatment of pain

Field of the invention

The present invention refers to the use of 5-HT₁ receptor agonists for the treatment of pain and the symptoms of pain, especially certain subtypes of pain like neuropathic pain and inflammatory pain and symptoms involving allodynia and hyperalgesia, the prevention or the prophylaxis of pain and the symptoms of pain, especially certain subtypes of pain like neuropathic pain and inflammatory pain and symptoms involving allodynia and hyperalgesia.

Background of the invention

The treatment of pain conditions is of great importance in medicine. There is currently a world-wide need for additional pain therapy. The pressing requirement for a specific treatment of pain conditions or as well a treatment of specific pain conditions which is right for the patient, which is to be understood as the successful and satisfactory treatment of pain for the patients, is documented in the large number of scientific works which have recently and over the years appeared in the field of applied analgesics or on basic research on nociception.

PAIN is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 210). Even though pain is always subjective its causes or syndromes can be classified.

Especially neuropathic pain which in the past years has developed into a major health problem in broad areas of the population needs a very specific treatment, especially considering that any treatment of neuropathic pain is extremely sensitive to the causes behind the pain, be it the disease ultimately causing it or the mechanistic pathway over which it develops.

Hyperalgesia and allodynia account for clinically relevant states such as inflammation and neuropathic pain. These conditions are characterized by alterations of pain perception that include enhanced sensitivity to noxious stimuli (hyperalgesia) and abnormal pain sensitivity to previously non-painful stimuli (allodynia). From the mechanistic point of view, the relationship between central sensitization and these pain
associated behaviors, hyperalgesia and allodynia, has been recognized. Central sensitization is a term coined to describe the increased excitability of CNS nociceptive neurons triggered by persistent input or peripheral injury (Woolf, 1983). In fact, many popular models of post-injury hypersensitivity revolve around the notion of central sensitization whereby injury-evoked afferent discharges cause spinal cord neurons to reduce their stimulation threshold, to expand their receptive fields and to increase their responsiveness to different peripheral drives (Woolf, 1983; Hylden et al., 1989; Woolf and Thompson 1991; Coderre and Katz, 1997).

Many different neurotransmitter systems, ion channels and enzymes have been implicated in pain transmission, processing and controlling. Among them, serotonin (5-hydroxytryptamine [5-HT]), produced by central and peripheral serotonergic neurons but also by platelets and mast cells after tissue injury, has been described to exert algesic or analgesic effects depending on the site of action and the receptor subtype it acts on (Eide and Hole, 1993; Millan, 2002). At the peripheral level, after nerve injury, 5-HT is released in increased amounts and interacts with different 5-HT receptors present on C-fibers (Sommier, 2004). Acting in combination with other inflammatory mediators, 5-HT may ectopically excite and sensitize afferent nerve fibers, thus contributing to peripheral sensitization and hyperalgesia following inflammation and nerve injury (Beck and Handwerker, 1974; Obata et al., 2000). The central serotonin system has also been the subject of considerable research over the last twenty years. Both 5-HT and noradrenaline (NE)-mediated pathways of descending inhibition have been described extensively. These monoaminergic tracts arise from the midbrain and brainstem and terminate on the spinal cord to suppress sensory transmission and consequently produce analgesia. However, descending pathways projecting to the dorsal horn not only suppress (descending inhibition) but they may also potentiate (descending facilitation) nociceptive messages. In the case of the descending 5-HT system, both inhibition and facilitation have been described depending on the 5-HT receptor involved and on their divergent neuronal localization (Millan, 2002; Suzuki et al., 2004; Oyama et al., 1996).

Different drugs acting on the central 5-HT system have been investigated and/or used as analgesics. Among them, antidepressants are frequently used as adjuvants for analgesic treatment of pain. A significant amount of evidence supports the use of tricyclic antidepressants (TCAs) and other 5-HT and NE reuptake inhibitors (SNRIs) in the management of chronic pain, but because of their effects on multiple systems, they are associated with numerous undesirable side effects (Carter and Sullivan, 2002;
Mattia et al., 2002; Barkin and Barkin, 2005). The newer selective 5-HT reuptake inhibitors (SSRIs), having only 5-HT-receptor-mediated side effects, have not been thoroughly studied but they seem to be less efficacious than TCAs and SNRIs in treating chronic pain (Barkin and Fawcett, 2000; Bomholt et al., 2005; Briley, 2004; Maizels and McCarberg, 2005; Sindrup et al., 2005; Stahl et al., 2005). In addition, available data suggest that increased 5-HT levels do not invariably inhibit nociceptive processing. Rather, it elicits a spectrum of pro- and antinociceptive actions that are receptor-dependent and a function of stimulus quality and modality (Millan, 2002; Suzuki et al., 2004).

As reviewed by Millan (2002), different 5-HT receptors have been implicated in analgesia. Among them, 5-HT1A, 5-HT1B/1D, 5-HT2A, 5-HT2C and 5-HT3 have received most attention (Eide and Hole, 1993; Oyama et al., 1996; Obata et al., 2000; Kayser et al., 2002; Colpaert, 2006).

A recent Immunocytochemical study investigating 5-HT7 receptor distribution at the lumbar level revealed that 5-HT7 immunolabelling is localized mainly in the two superficial laminae of the dorsal horn and in small and medium-sized dorsal root ganglion cells, which is consistent with a predominant role in nociception (Doly et al., 2005). In rat and human dorsal root ganglia, the presence of 5-HT7 receptor messenger RNA has also been detected (Pierce et al., 1996, 1997). Electron microscopic examination of the dorsal horn revealed three main localizations: 1) a postsynaptic localization on peptidergic cell bodies in laminae I-III and in numerous dendrites; 2) a presynaptic localization on unmyelinated (presumably of primary afferent origin) and thin myelinated peptidergic fibers (from intrinsic cells); and 3) a localization on astrocytes in lamina I and II (Doly et al., 2005).

Regarding its function, a possible pronoceptive role of peripheral and spinal 5-HT7 receptors has been described in the formalin test based on local paw and intrathecal administration of 5-HT or the non-selective 5-carboxamidotryptamine (5-CT) serotonergic agonist and reversion of their effects by the 5-HT7 receptor antagonist SB-269970 (Rocha-González, 2005).

As especially neuropathic pain has developed into a major health problem in broad areas of the population and needs a very specific treatment, it was the underlying problem solved by this invention to find new ways of treating pain, especially neuropathic pain.
So, the main object of this invention is the use of a compound binding to the 5-HT\textsubscript{7} receptor and acting as an agonist in the production of a medicament for the treatment of pain.

This/these compound/s may be in neutral form, the form of a base or acid, in the form of a salt, preferably a physiologically acceptable salt, in the form of a solvate or of a polymorph and/or in the form of in the form of its racemate, pure stereoisomers, especially enantiomers or diastereomers or in the form of mixtures of stereoisomers, especially enantiomers or diastereomers, in any suitable ratio.

While working on compounds binding to the 5-HT\textsubscript{7} receptor and which act there as agonists it was surprisingly found out that these compounds could act on the treatment of pain, especially neuropathic pain, but also inflammatory pain with a high potency. This was all the more surprising as there was a clear tendency in the art to expect that it was the compounds binding to the 5-HT\textsubscript{7} receptor but which act there as antagonists would be the ones usable for the treatment of pain, especially neuropathic pain. Clearly this was found here as not being the case, especially not for neuropathic pain.

"Treating" or "treatment" as used in this application are defined as including the treatment of the symptoms of pain, especially neuropathic pain, especially certain subtypes of neuropathic pain - as well as treatment of the disease or disease consequences causing the symptoms, the prevention or the prophylaxis of the symptoms of pain, especially neuropathic pain, especially certain subtypes of neuropathic pain - as well as the prevention or the prophylaxis of the disease or disease consequences causing the symptoms. Preferably "treating" or "treatment" as used in this application are defined as including the treatment of the symptoms of pain, especially neuropathic pain, especially certain subtypes of neuropathic pain - as well as treatment of the disease consequences causing the symptoms, the prevention or the prophylaxis of the symptoms of pain, especially neuropathic pain, especially certain subtypes of neuropathic pain - as well as the prevention or the prophylaxis of the disease consequences causing the symptoms. Most preferably "treating" or "treatment" as used in this application are defined as including the treatment of the symptoms of pain, especially of neuropathic pain, especially certain subtypes of neuropathic pain, and the prevention or the prophylaxis of the symptoms of pain, especially of neuropathic pain, especially certain subtypes of neuropathic pain.
The superfamily of serotonin receptors (5-HT) includes 7 classes (5-HT₁-5-HT₇) encompassing 14 human subclasses that are described in many positions in literature especially in D. Hoyer, et al., Neuropharmacology, 1997, 36, 419. Thus:

"The 5-HT₇ receptor" as used in this application is well known and defined.
"The 5-HT₁A receptor" as used in this application is well known and defined.
All other "5-HT receptors" as used in this application are well known and defined.

"Compound binding to the 5-HT₇ receptor" as used in this application is/are defined as having a Kᵢ Value in their binding to the 5-HT₇-receptor ≤ 2 μM.

Assays that may be used for determining the affinity and selectivity of a 5-HT₇ receptor agonist and/or other affinities to 5-HT receptors are well known in the art and especially measuring the affinities to these receptors are offered by service companies like CEREP (128, rue Danton, 92500 Rueil. Malmaison, France) or MDS Pharma Services (Rosa de Lima 1bis, 2º 28290 Las Matas (Madrid), Spain). It is possible to classify a compound with 5-HT receptor affinity as agonist (also as inverse agonist or antagonist) according to the reference of S. M. Stahl, Essential Psychopharmacology, Neuroscientific basis and practical applications, Ed. Cambridge, 1996, Chapter 3. The respective part of the literature is hereby incorporated by reference and forms part of the disclosure.

An "Agonist" is defined as a compound that binds to a receptor and has an intrinsic effect, and thus, increases the basal activity of a receptor when it contacts the receptor. Full agonists show the maximum effect like in this case 5CT or AS-19 on the 5-HT₇ receptor, whereas a partial agonist like MSD-5a is giving less (e.g. 80%) of the response of the full agonist as a maximum.

The term "salt" is to be understood as meaning any form of the active compound according to the invention in which this assumes an ionic form or is charged and is coupled with a counter-ion (a cation or anion) or is in solution. By this are also to be understood complexes of the active compound with other molecules and ions, in particular complexes which are complexed via ionic interactions.

The term "physiologically acceptable salt" is understood in particular, in the context of this invention, as salt (as defined above) formed either with a physiologically tolerated acid, that is to say salts of the particular active compound with inorganic or organic acids which are physiologically tolerated - especially if used on humans and/or
mammals - or with at least one, preferably inorganic, cation which are physiologically tolerated - especially if used on humans and/or mammals. Examples of physiologically tolerated salts of particular acids are salts of: hydrochloric acid, hydrobromic acid, sulfuric acid, hydrobromide, monohydrobromide, monohydrochloride or hydrochloride, methiodide, methanesulfonic acid, formic acid, acetic acid, oxalic acid, succinic acid, malic acid, tartaric acid, mandelic acid, fumaric acid, lactic acid, citric acid, glutamic acid, hippuric acid picric acid and/or aspartic acid. Examples of physiologically tolerated salts of particular bases are salts of alkali metals and alkaline earth metals and with NH₄.

The term “solvate” according to this invention is to be understood as meaning any form of the active compound according to the invention in which this compound has attached to it via non-covalent binding another molecule (most likely a polar solvent) especially including hydrates and alcoholates, e.g. methanolate.

In one embodiment of the current invention the following proviso applies to the compounds used in this invention:

- with the proviso that the compound epinastin is excluded.

In one embodiment of the current invention the following proviso applies to the compounds used in this invention:

- with the proviso that the compound 8-OH-DPAT (8-hydroxy-dipropylaminotetralin) is excluded.

Some known subgroups of pain are acute pain, chronic pain, visceral pain and including also headaches, especially migraine. A subgroup of pain being of special interest in connection with this invention is neuropathic pain, which is the pain treated as highly preferred embodiment of the invention. Another subgroup of pain being also in the focus of this invention is inflammatory pain.

Therefore a preferred use according to the invention is the use of a compound binding to the 5-HT₇ receptor and acting as an agonist in the production of a medicament for the treatment of pain in which the pain is neuropathic pain, especially hyperalgesia or allodynia.

Another preferred use according to the invention is the use of a compound binding to the 5-HT₇ receptor and acting as an agonist in the production of a medicament for the
treatment of pain in which the pain is neuropathic pain involving hyperalgesia or allodynia.

Another preferred use according to the invention is the use of a compound binding to the 5-HT₇ receptor and acting as an agonist in the production of a medicament for the treatment of pain in which the pain is hyperalgesia.

Another preferred use according to the invention is the use of a compound binding to the 5-HT₇ receptor and acting as an agonist in the production of a medicament for the treatment of pain in which the pain is allodynia.

Another preferred use according to the invention is the use of a compound binding to the 5-HT₇ receptor and acting as an agonist in the production of a medicament for the treatment of pain in which the pain is inflammatory pain.

“Neuropathic pain” is defined by the IASP as “pain initiated or caused by a primary lesion or dysfunction in the nervous system” (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 210). For the purpose of this invention included under this heading or to be treated as synonymous is “Neurogenic Pain” which is defined by the IASP as “pain initiated or caused by a primary lesion, dysfunction or transitory perturbation in the peripheral or central nervous system”. One of the many reasons causing neuropathic pain is diabetes, especially diabetes II.

According to the IASP “allodynia” is defined as “a pain due to a stimulus which does not normally provoke pain” (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 210).

According to the IASP “hyperalgesia” is defined as “an increased response to a stimulus which is normally painful (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 211).

In a preferred embodiment of the invention the medicament is used for the treatment of neuropathic pain - or its subgroups - in which the stimulus evoking the pain – especially the neuropathic pain - is mechanical.
In another embodiment of the invention the medicament is used for the treatment of neuropathic pain - or its subgroups - in which the stimulus evoking the pain – especially the neuropathic pain - is thermal.

In another preferred embodiment of the invention the medicament is used for the treatment of inflammatory pain in which the stimulus evoking the pain – especially the inflammatory pain - is mechanical.

In another embodiment of the invention the medicament is used for the treatment of inflammatory pain in which the stimulus evoking the pain – especially the inflammatory pain - is thermal.

In another preferred embodiment of the use according to the invention the neuropathic pain, is selected from central pain, hyperpathia, peripheral neuropathic pain – especially peripheral somatic or cephalic neuropathic pain - or peripheral neurogenic pain, causalgia, hyperesthesia, neuralgia, neuritis, or neuropathy.

According to the IASP "central pain" is defined as "a pain initiated or caused by a primary lesion or dysfunction in the central nervous system" (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 211).

According to the IASP "causalgia" is defined as "a syndrome of sustained burning pain, allodynia and hyperpathia after a traumatic nerve lesion, often combined with vasomotor and sudomotor dysfunction and later trophic changes" (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 210).

According to the IASP "hyperesthesia" is defined as "increased sensitivity to stimulation, excluding the senses" (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 211).

According to the IASP "neuralgia" is defined as "Pain in the distribution of a nerve or nerves" (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 212).

According to the IASP "neuritis" is defined as "Inflammation of a nerve or nerves" (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 212).
According to the IASP "neuropathy" is defined as "a disturbance of function or pathological change in a nerve: in one nerve mononeuropathy, in several nerves mononeuropathy multiplex, if diffuse and bilateral, polyneuropathy" (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 212).

According to the IASP "hyperpathia" is defined as "a painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold" (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 212).

The IASP draws the following difference between "allodynia", "hyperalgesia" and "hyperpathia" (IASP, Classification of chronic pain, 2nd Edition, IASP Press (2002), 212):

<table>
<thead>
<tr>
<th>Allodynia</th>
<th>Lowered threshold</th>
<th>Stimulus and response mode differ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperalgesia</td>
<td>Increased response</td>
<td>Stimulus and response rate are the same</td>
</tr>
<tr>
<td>Hyperpathia</td>
<td>Raised threshold; Increased response</td>
<td>Stimulus and response rate may be the same or different</td>
</tr>
</tbody>
</table>

Especially preferred embodiments of the invention encompass the use of a compound with a very specific binding to the 5-HT7 receptor being in its binding profile more specific in its affinity (thus showing a lower Ki) to the 5-HT7 receptor than in its affinity to other 5-HT Receptors.

Therefore another preferred use according to the invention is the use of a compound binding to the 5-HT7 receptor and acting as an agonist, wherein the compound binding to the 5-HT7 receptor is binding with a higher affinity – expressed as a lower Ki-value – to the 5-HT7 receptor than to the 5-HT1A receptor; especially binding with an affinity higher by a factor of at least 10, preferably with an affinity higher by a factor of at least 30, more preferably with an affinity higher by a factor of at least 50, most preferably with an affinity higher by a factor of at least 100.
By way of exemplifying the above paragraph an compound X – specific to the 5-HT\textsubscript{7} receptor -, assumed to have an affinity – expressed as a Ki value - of 1 nM and an affinity to the 5HT\textsubscript{1A} receptor of a Ki value of 42 nM.

Another preferred use according to the invention is the use of a compound binding to the 5-HT\textsubscript{7} receptor and acting as an agonist, wherein the compound binding to the 5-HT\textsubscript{7} receptor is binding with a higher affinity – expressed as a lower Ki-value – to the 5-HT\textsubscript{7} receptor than to the any other 5-HT receptor; especially binding with an affinity higher by a factor of at least 10, preferably with an affinity higher by a factor of at least 30, more preferably with an affinity higher by a factor of at least 50, most preferably with an affinity higher by a factor of at least 100.

Another preferred use according to the invention is the use of a compound binding to the 5-HT\textsubscript{7} receptor and acting as an agonist, wherein the compound binding to the 5-HT\textsubscript{7} receptor is binding to the 5-HT\textsubscript{7} receptor with a Ki-value lower than 1000 nM, preferably lower than 200 nM, more preferably lower than 100 nM, even more preferably lower than 50 nM, very preferably lower than 25 nM, most preferably lower than 10 nM.

Another preferred use according to the invention is the use of a compound binding to the 5-HT\textsubscript{7} receptor and acting as an agonist, wherein the compound binding to the 5-HT\textsubscript{7} receptor is binding to the 5-HT\textsubscript{7} receptor with a Ki-value lower than 1000 nM, preferably lower than 200 nM, more preferably lower than 100 nM, even more preferably lower than 50 nM, very preferably lower than 25 nM, most preferably lower than 10 nM

and

is binding with a higher affinity – expressed as a lower Ki-value – to the 5-HT\textsubscript{7} receptor than to the 5-HT\textsubscript{1A} receptor; especially binding with an affinity higher by a factor of at least 10, preferably with an affinity higher by a factor of at least 30, more preferably with an affinity higher by a factor of at least 50, most preferably with an affinity higher by a factor of at least 100.

Another preferred use according to the invention is the use of a compound binding to the 5-HT\textsubscript{7} receptor and acting as an agonist, wherein the compound binding to the 5-HT\textsubscript{7} receptor is binding to the 5-HT\textsubscript{7} receptor with a Ki-value lower than 1000 nM,
preferably lower than 200 nM, more preferably lower than 100 nM, even more preferably lower than 50 nM, very preferably lower than 25 nM, most preferably lower than 10 nM

and

is binding with a higher affinity – expressed as a lower Ki-value – to the 5-HT\textsubscript{7} receptor than to any other 5-HT receptor; especially binding with an affinity higher by a factor of at least 10, preferably with an affinity higher by a factor of at least 30, more preferably with an affinity higher by a factor of at least 50, most preferably with an affinity higher by a factor of at least 100.

Another preferred use according to the invention is the use of a compound binding to the 5-HT\textsubscript{7} receptor and acting as an agonist, wherein the compound is either a full or partial agonist.

In another preferred use according to the invention the compound used is either AS-19 or MSD5a, especially AS-19, optionally in the form of its racemate, pure stereoisomers, especially enantiomers or diastereomers or in the form of mixtures of stereoisomers, especially enantiomers or diastereomers, in any suitable ratio; in the form shown or in form of the acid or base or in form of a salt, especially a physiologically acceptable salt, or in form of a solvate, especially a hydrate.

AS-19 [(2S)-(+)\textsubscript{-}\textsubscript{8}-(1,3,5-trimethylpyrazolin-4-yl)-2-(dimethylamino) tetralin] is defined by below sketched formula and is commercially available (e.g. through TOCRIS).
MSD-5a (corresponds to compound 5a in Thomson et al., 2004; Merck Sharp & Dohme Research Laboratories) [2-(6-phenylpyridin-2-ylthio)-N,N-dimethyl ethanamine] is described in this literature including its synthesis.

Any use of a compound that is a prodrug of a compound used according to the invention - especially AS-19 and MSD-5a - is within the scope of the invention. The term "prodrug" is used in its broadest sense and encompasses those derivatives that are converted in vivo to the compounds of the invention. Such derivatives would readily occur to those skilled in the art, and include, depending on the functional groups present in the molecule and without limitation, the following derivatives of the present compounds: esters, amino acid esters, phosphate esters, metal salts sulfonate esters, carbamates, and amides. Examples of well known methods of producing a prodrug of a given acting compound are known to those skilled in the art and can be found e.g. in Krogsgaard-Larsen et al. "Textbook of Drug design and Discovery" Taylor & Francis (April 2002).

Unless otherwise stated, the compounds used according to the invention - especially AS-19 and MSD-5a - are also meant to include compounds which differ only in the presence of one or more isotopically enriched atoms. For example, compounds having the present structures except for the replacement of a hydrogen by a deuterium or tritium, or the replacement of a carbon by $^{13}$C- or $^{14}$C-enriched carbon or $^{15}$N-enriched nitrogen are within the scope of this invention.

In human therapeutics, the dose administered can be quite low depending on the route of administration. Generally an effective administered amount of a compound of the invention will depend on the relative efficacy of the compound chosen, the severity of the disorder being treated and the weight of the sufferer. However, active compounds will typically be administered once or more times a day for example 1, 2, 3 or 4 times daily, with typical total daily doses in the range of from 0.1 to 1000 mg/kg/day.

Any medicament according to the invention contains the active ingredient as well as optionally at least one auxiliary material and/or additive and/or optionally another active ingredient.

The auxiliary material and/or additive can be specifically selected from conserving agents, emulsifiers and/or carriers for parenteral application. The selection of these
auxiliary materials and/or additives and of the amounts to be used depends upon how the pharmaceutical composition is to be applied. Examples include here especially parenteral like intravenous subcutaneous or intramuscular application formulations but which could also be used for other administration routes.

Routes of administration can include intramuscular injection, intravenous injection, subcutaneous injection, sublingual, bucal, patch through skin, oral ingestion, implantable osmotic pump, collagen implants, aerosols or suppository.

Included in this invention are especially also methods of treatments of a patient or a mammal, including men, suffering from pain, especially neuropathic pain using a compound binding to the 5HT7 receptor and acting as an agonist.

**Pharmacological methods:**

**Method 1: Binding and functionality**

**Binding**

Binding affinities were determined in radioligand binding assays according to their standard assays protocols. In case 1, the assays included in the LeadProfilingScreen package plus 5-HT1 non-selective, 5-HT2 non-selective, 5-HT2B, 5-HT2C, 5-HT4, 5-HT5A, 5-HT6 and 5-HT7 were performed. In case 2, assays included in the High-throughput profile plus 5-HT1D, 5-HT2B, 5-HT4E were performed. For AS-19 further assays were carried out to complete its selectivity profile for serotonin receptors, including 5-HT1B, 5-HT1D, 5-HT2A and 5-HT4E.

**Functionality**

cAMP measurements were performed by using a system based on Homogeneous Time Resolved Fluorescense (HTRF) (Gabriel et al., 2003). This technology allows the direct measurement of cAMP on HEK-293F cells that stably expressed human 5-HT7 receptors. The principle of this assay is based on competition between cAMP produced by cells and cAMP-XL665 conjugate for the binding with monoclonal anti-cAMP-cryptate conjugate. The HTRF cAMP kit from CisBio was used according to the manufacturer's directions. The experimental procedure was performed as stated below. After overnight serum-free incubation, suspended cells (20,000 cells per well) were added to 96-well culture plates in incubation buffer composed of Ham's F12 (Gibco) plus 1 mM 3-isobutyl-1- methyl-xanthine (IBMX; Sigma) and 20 μM pargyline (Sigma).
For agonist and antagonist experiments, 2 μl of either compound or vehicle was added to each well on 40 μl of cell suspension at different concentrations, preincubating plates for 10 min at room temperature after compound addition. Afterwards, 10 μl of either vehicle or 5-HT was added incubating for 30 min at 37 °C. The reaction was stopped lysing the cells with a mixture of 25 μl of cryptate and 25 μl of XL-665 prepared in the lysis buffer supplied by the manufacturer. Plates were incubated for an additional hour at room temperature and read at 665 nm/620 nm using a RubyStar Plate reader (BMG LabTech).

**Method 2: Capsaicin test**

**MATERIAL AND METHODS**

**Animals**

Male CD1 mice aged from 6 to 8 weeks old were used in these studies. Animals were housed in groups of five, provided with food and water ad libitum and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and light in 12 h cycles (on at 07:00 h and off at 19:00 h). Experiments were carried out in a soundproof and air-regulated experimental room. The number of mice per group in each individual experiment was 16.

**Drugs**

Capsaicin (8-methyl-N-vanillyl 6-nonamide) was dissolved in 1% DMSO (vehicle). Drugs used for treatments were: AS-19 [(2S)-(+)8-(1,3,5-trimethylpyrazolin-4-yl)-2-(dimethylamino) tetralin], MSD-5a (corresponds to compound 5a in Thomson et al., 2004; Merck Sharp & Dohme Research Laboratories) [2-(6-phenylpyridin-2-ylthio)-N,N_dimethyl ethanamine], SB-258719 [(R)-3,N-Dimethyl-N-(1-methyl-3-(4-methylpiperidin-1-yl) propyl) benzene sulfonamide], SB-269970 [(R)-3-(2-(2-(4-Methylpiperidin-1-yl) ethyl) pyrrolidine-1-sulfonyl) phenol], WAY-100635 [(N-(2-(4-(2-methoxyphenyl)-1-piperazinyl) ethyl)-N-(2-pyridinyl) cyclohexane carboxamide] and F-13640 [(3-Chloro-4-fluoro-phenyl)-[4-fluoro-4-[(5-methyl-pyridin-2-ylmethyl)-amino]-methyl]piperidin-1-yl] methanone]. AS-19 was dissolved in 1% DMSO and the rest of compounds were prepared as aqueous solutions. Doses were calculated based on the molecular weight of the free base. All the compounds and vehicles (1% DMSO or physiological saline) were administered in a volume of 5 ml/kg through the subcutaneous (s.c.) route. When
two compounds were co-administered, they were injected s.c. in opposite flanks of the mice.

**Capsaicin model of neurogenic pain**

Subplantar capsaicin injection initially evokes a nocifensive behavior that is characterized by lifting and guarding the injected paw and typically lasts up to 5 min following injection. Afterwards, hypersensitivity to both thermal and mechanical stimuli is evidenced (Gilchrist et al., 1996). In this study, sensitization by subplantar capsaicin injection was used to assess the effect of several 5-HT\(_{7}\) ligands on mechanical allodynia.

Mice were injected with capsaicin (1\(\mu\)g or 4 \(\mu\)g in 20\(\mu\)l of 1\% DMSO) or 1\% DMSO (vehicle) into the mid-plantar surface of the right hind paw. Drug treatments with 5-HT\(_{7}\) ligands were done 30 min before capsaicin injection and withdrawal latencies to a mechanical stimulus were determined 15 min after capsaicin injection (Fig. 1).

**Evaluation of mechanical allodynia: automatic von Frey test**

Mechanical allodynia was quantified by measuring the hind paw withdrawal response latency to von Frey filament stimulation. Mice were placed into compartment enclosures in a test chamber with a framed metal mesh floor and allowed to acclimate to their environment for 120 min before testing. During this time the animal initially demonstrated exploratory behavior, but subsequently stopped exploring and stood quietly with only occasional bouts of grooming. The mechanical stimulus was applied to the plantar surface of the right, capsaicin-injected hind paw from below the mesh floor by using an automated testing device (dynamic plantar aesthesiometer; Ugo Basile, Italy) that lifts a straight filament (0.5 mm diameter). The filament exerted a fixed upward pressure (1 g or 0.25 g) on the plantar surface. When the animal withdrew its hind paw, the mechanical stimulus automatically stopped and the latency time was recorded. Latency was defined as the time from the onset of exposure to the filament to the cessation of the pressure when the sensor detected the withdrawal of the paw. A cut-off latency of 60 sec was imposed. Paw withdrawal latencies were measured in triplicate for each animal.

**Experimental protocol**

When fixed at 1 g, the pressure exerted by the filament is insufficient to induce timely pain responses in normal control mice (in the absence of capsaicin) and thus prompt withdrawal responses found 15 min after sensitization with 1 \(\mu\)g capsaicin reflect
mechanical allodynia. This approach enabled us to evaluate the possible antiallodynic effect of 5-HT\textsubscript{7} ligands. Alternatively, in some experiments, only 4 ng (instead of 1\mu g) of capsaicin were injected into the paw and the pressure exerted by the filament was reduced to 0.25 g (instead of 1 g). Mice receiving this minor amount of capsaicin (250 times less than those used to induce allodynia) did not display allodynia when mechanically stimulated with a low upward pressure of 0.25 g. This approach was used to test the possibility that treatments could exert a proalldyenic effect. The Figure 1 summarizes the two different experimental approaches carried out in this study.

**Data analysis**

Data are presented as mean paw withdrawal latency responses in sec ± S.E.M. Capsaicin-injected hind paw withdrawal responses to mechanical stimulation of treated mice were compared with those of untreated (vehicle-treated) mice. Statistical analysis to test intergroup comparisons was made using an initial one-way analysis of variance followed by the Newman-Keuls multiple comparison test. The level of significance was set at \( p < 0.05 \).

**Method 3: Partial sciatic nerve ligation**

**MATERIAL AND METHODS**

**Animals**

Male CD1 mice aged from 6 to 8 weeks old were used in these studies. Animals were housed in groups of five, provided with food and water ad libitum and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and light in 12 h cycles (on at 07:00 h and off at 19:00 h). Experiments were carried out in a soundproof and air-regulated experimental room.

**Drugs**

Drugs used for treatments were: AS-19 [(2S)-(+)8-(1,3,5-trimethylpyrazolin-4-yl)-2-(dimethylamino) tetrain], MSD-5a (corresponds to compound 5a in Thomson et al., 2004; Merck Sharp & Dohme Research Laboratories) [2-(6-phenylpyridin-2-ythio)-N,N-dimethyl ethanamine], SB-258719 [(R)-3,N-Dimethyl-N-(1-methyl-3-(4-methylpiperidin-1-yl) propyl) benzene sulfonamide]. AS-19 was dissolved in 1% DMSO in the free base form and saline with the dihydrochloride salt. The rest of compounds were prepared as aqueous solutions. Doses were calculated based on the molecular weight of the free
base. All the compounds and vehicles (1% DMSO or physiological saline) were administered in a volume of 5 ml/kg through the subcutaneous (s.c.) route. When two compounds were co-administered, they were injected s.c. in opposite flanks of the mice.

**Surgery**

The partial sciatic nerve ligation model was used to induce neuropathic pain, according to the method previously described (Seltzer et al., 1990; Malmberg and Basbaum, 1998). This model consists of partial injury to the sciatic nerve at mid-thigh level. All surgical procedures for nerve injury were performed under isoflurane (IsoFlo®, Abbott-Laboratorios Dr. Esteve, S.A., Barcelona, Spain) anesthesia (induction: 3%; surgery: 2%). Briefly, mice were anaesthetized and the common sciatic nerve was exposed at the level of the mid-thigh of the right hind paw. Partial nerve injury was produced at about 1 cm proximal to the nerve trifurcation by tying a tight ligature around approximately 33 to 50% the diameter of the sciatic nerve using 9-0 non-absorbable virgin silk suture (Alcon surgical, Texas, USA). Ligature enclosed the outer 1/3-1/2 sciatic nerve whereas the rest of the nerve (inner 2/3-1/2) was lefted "uninjured". The muscle was then stitched with 6-0 silk suture and the skin incision was closed with wound clips. Control, sham-operated mice underwent the same surgical procedure and the sciatic nerve was exposed, but not ligated. After surgery, animals were allowed to recover for 5 days prior to initiation of nociceptive behavioral tests (see experimental protocol below).

**Nociceptive behavioral tests**

Allodynia to mechanical stimulus and hyperalgesia to noxious thermal stimulus were used as outcome measures of neuropathic pain and as indicators of the possible antinociceptive effect of drug treatments.

**Evaluation of mechanical allodynia: automatic von Frey test**

Mechanical allodynia was quantified by measuring the tactile threshold for hind paw withdrawal following von Frey filament stimulation. The day of the test, mice were placed into compartment enclosures in a test chamber with a framed metal mesh floor and allowed to acclimate to their environment for 1 h before testing. The mechanical stimulus was applied to the plantar surface of the right hind paw from below the mesh floor by using an automated testing device (dynamic plantar aesthesiometer; Ugo Basile, Italy) that lifts a straight filament (0.5 mm diameter). The filament exerted an increasing upward pressure on the plantar surface. The pressure increased linearly
with time (0.1g/s; from 0 to 5 g in 50 s) until the mouse withdrew its paw. When the animal withdrew its hind paw, the mechanical stimulus automatically stopped and the force was recorded as the mechanical threshold. Thus, the mechanical threshold was defined as the pressure at which the sensor detected the withdrawal of the paw. A cut-off threshold of 5 g (50 s) was imposed. Paw withdrawal latencies were measured in triplicate for each animal.

**Evaluation of mechanical allodynia: manual von Frey test.**
Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation, as previously reported (Chaplan et al., 1994). The filament of 0.4 g was first used. Then, the strength of the next filament was decreased when the animal responded or increased when animal did not respond. This up-down procedure was stopped four measures after the first change in animal responding and the threshold of response was calculated. Clear paw withdrawal, shaking was considered as a nociceptive-like response.

**Evaluation of thermal hyperalgesia: plantar test**
Thermal hyperalgesia was assessed using the plantar test by determination of the withdrawal latencies to a thermal stimulus following a modification of the test described by Hargreaves et al. (1988). The day of the test, mice were placed in a plantar test device (Plantar test, Ugo Basile, Italy) and allowed to acclimate to their environment for 1 h before testing. The plantar test device consisted of a plastic cubicle with a glass floor through which a mobile infrared photobeam was applied onto the plantar surface of the right hind paw. Latency time to withdrawal from the thermal stimulus was determined. The intensity of the infrared photobeam was adjusted to produce mean response latencies in control untreated mice of approximately 8 to 12 seconds. The response latency was determined using a timer linked to the photodiode motion sensors in the plantar test device. Response latency was defined as the time from the onset of exposure to the infrared photobeam to the cessation of the photobeam when the photodiode motion sensor detected the withdrawal of the paw of the mouse. Paw withdrawal latencies were measured in triplicate for each animal and the mean value was used for analysis. A cut-off latency of 20 seconds was imposed to avoid tissue damage.

**Experimental protocol: Latin square design.**
The effectiveness of acute treatments with 5-HT7 ligands compared to vehicle on the expression of neuropathic pain was investigated based on the following experimental
protocol. Mice were habituated for 1 h to the environment of the different experimental tests (either von Frey or plantar test, depending on the experiment) during 4 days. After the habituation period, pre-injury baseline responses in each test were established during 2 consecutive days. One day after baseline measurements, nerve injury or sham operation was induced as previously described. Mice were then tested in either the von Frey or the plantar test on day 5 after the surgical procedure to monitor post-injury responses. Post-injury baseline responses of mice treated with vehicle were obtained on day 10 (basal pre-treatment). On days 11-13 mice received treatment with three different doses of 5-HT7 ligands following a Latin square design. Finally, on day 14 after surgery, baseline responses in vehicle-treated mice (basal post-treatment) were evaluated as an internal control to ensure that mechanical allodynia and thermal hyperalgesia were not influenced by previous treatments in the Latin square design. Behavioral evaluation was always done 30 min after treatment with either vehicle or 5-HT7 ligands. Figure 15 summarizes the chronology of the experimental protocol.

Experimental protocol: Single dose design.

Mice were habituated for 2 h to the environment of the different experimental tests (either manual von Frey or plantar test, depending on the experiment) during 1-2 days. After the habituation period, measurements in each test were established after 1 h habituation by means of the manual von Frey or the plantar test, 30 and 45 min, respectively, after administration of the vehicle or the tested compounds. One day after baseline measurement, nerve injury was induced as previously described and 5 days later post-injury baseline values were obtained in the mentioned tests. On day 10 post-injury mice received vehicle and measurements were done to obtain pre-treatment basal values. The day of the test, on day 11 post-injury, measurements in drug-treated mice were done (Fig. 16).

Data analysis

Data were primarily presented as pressure (grams) required for reaching the threshold of response (paw withdrawal) in the von Frey test or as latency (seconds) of paw withdrawal in the plantar test. In some cases, data were presented as percentages respect to pre-treatment baseline values. Values were always mean ± S.E.M.

Data obtained in the von Frey and plantar tests in treated mice were compared with those of untreated (vehicle-treated) mice. Statistical analysis to test intergroup comparisons was made using an initial one-way analysis of variance followed by the post hoc Newman-Keuls multiple comparison test. Differences were considered
significant if the probability of error was less than 5% (the level of significance was set at $p < 0.05$).

REFERENCES


Stahl SM, Grady MM, Moret C, Briley M. (2005) SNRIs: their pharmacology, clinical efficacy, and tolerability in comparison with other classes of antidepressants. CNS Spectr, 10(9):732-747


Method 4: Chronic constriction injury of the sciatic nerve (CCI-SN)

MATERIAL AND METHODS

Animals
Adult male Sprague-Dawley rats (175-200 g on arrival) were housed in a temperature controlled environment (22 ± 1°C) with a 12 h light /12 h dark cycle. Food and water were available ad libitum. Rats were allowed to habituate to the housing facilities for at least 1 week before surgery. Ethical guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (1983) were adhered to in these studies.

Drugs
Drugs used for treatments were: AS-19 [(2S)-(+)8-(1,3,5-trimethylpyrazolin-4-yl)-2-(dimethylamino) tetrain], MSD-5a (corresponds to compound 5a in Thomson et al., 2004; Merck Sharp & Dohme Research Laboratories) [2-(6-phenylpyridin-2-ythio)-N,N-dimethyl ethanamine], SB-269970 [(R)-3-(2-(4-Methylpiperidin-1-yl) ethyl) pyrrolidine-1-sulfonlyl) phenol], SB-258719 [(R)-3,N-Dimethyl-N-(1-methyl-3-(4-
methyl[piperidin-1-yl] propyl) benzene sulfonamide]. All compounds were prepared as aqueous solutions. AS-19 and MSD-5a were administered by subcutaneous (s.c.) route, whereas SB-258719 and SB-269970 were administered through the intraperitoneal (i.p.) route. When two compounds were co-administered, one was injected s.c. and the other i.p.

**Surgery**

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and CCI-SN was made according to the method of Bennett and Xie (1988). Briefly, the right common sciatic nerve was exposed at mid-tight level by blunt dissection through the biceps femoris muscle. Proximal to the trifurcation, \( \approx 10 \) mm of nerve was freed from adhering tissue and four loose ligatures (5.0 chromic catgut) were tied around the sciatic nerve (\( \approx 1 \) mm apart). To obtain the desired degree of constriction, the criterion formulated by Bennett and Xie (1988) was applied: the ligatures reduced the diameter of the nerve by a just noticeable amount, and retarded, but did not interrupt, the epineurial circulation. In sham-operated control rats, an identical dissection was performed on the right side allowing the sciatic nerve to be freed from surrounding tissue, but not ligatured. Finally, muscle and skin were closed in layers with silk thread.

**Nociceptive test procedures**

Mechanical nociceptive thresholds, expressed as grams (g), were measured using the Randall-Sellito test (Ugo Basile analgesimeter; Bioseb, Chaville, France). An increasing pressure was applied to the nerve-injured hindpaw of unrestrained rats until paw withdrawal (PW) and then a squeak (i.e. the vocalization threshold when the paw was held under pressure) were obtained. As PW is a spinally coordinated reflex whereas vocalization is a supra-spinal integrated response (Kayser and Christensen, 2000), this test allowed assessment of the respective contribution of spinal versus supra-spinal mechanisms.

**REFERENCES**


**Method 5: Chronic constriction injury of the infraorbital nerve (CCI-ION)**

**MATERIAL AND METHODS**

**Animals**
Adult male Sprague-Dawley rats (175-200 g on arrival) were housed in a temperature controlled environment (22 ± 1°C) with a 12 h light /12 h dark cycle. Food and water were available ad libitum. Rats were allowed to habituate to the housing facilities for at least 1 week before surgery. Ethical guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (1983) were adhered to in these studies.

**Drugs**
Drugs used for treatments were: AS-19 [(2S)-(+) -8-(1,3,5-trimethylpyrazolin-4-yl)-2-(dimethylamino) tetralin], MSD-5a (corresponds to compound 5a in Thomson et al., 2004; Merck Sharp & Dohme Research Laboratories) [2-(6-phenylpyridin-2-ylthio)-N,N-dimethyl ethanamine], SB-269970 [(R)-3-(2-(2-(4-Methylpiperidin-1-yl) ethyl) pyrrolidine-1-sulfonyl) phenol], SB-258719 [(R)-3,N-Dimethyl-N-(1-methyl-3-(4-methylpiperidin-1-yl) propyl) benzene sulfonamide]. All compounds were prepared as aqueous solutions. AS-19 and MSD-5a were administered by subcutaneous (s.c.) route, whereas SB-258719 and SB-269970 were administered through the intraperitoneal (i.p.) route. When two compounds were co-administered, one was injected s.c. and the other i.p.

**Surgery**
After pentobarbital-induced anaesthesia, the head of the rat was fixed in a stereotaxic frame, and a mid-line scalp incision was made, exposing skull and nasal bone. The infraorbital part of the right infraorbital nerve was then exposed. The edge of the orbit, formed by the maxillary, frontal, lacrimal and zygomatic bones, was dissected free, and orbital contents were gently deflected to give access to the infraorbital nerve. The latter was then dissected free at its most rostral extent in the orbital cavity, just caudal to the
infraorbital foramen. Two chromic catgut (5-0) ligatures were tied loosely (with about 2 mm spacing) around the nerve (Vos et al., 1994; Kayser et al., 2002). Care was taken to avoid any interruption of the epineurial circulation. In sham-operated rats, the right infraorbital nerve was exposed, but it was not ligatured.

Nociceptive test procedures

In these rats with unilateral ligatures on the infraorbital nerve, sensitivity to mechanical stimulation in the ipsilateral vibrissal territory was assessed by determination of pressure threshold, delivered through von Frey filaments (Semmes-Weinstein monofilaments, Stoelting, Wood Dale, IL, USA), necessary to trigger defensive behavioural response (brisk withdrawal of the head; attack; escape reaction). For each stimulus filament (corresponding to a calibrated pressure of 0.217, 0.445, 0.745, 0.976, 2.35, 4.19, 6.00, 7.37 or 12.5 g), three consecutive applications (1 sec apart) were made in order to verify the stability of the response. In both sciatic nerve- and infraorbital nerve- ligatured rats, threshold values were determined first two days before surgery, then 14 days later, at a time when hyperresponsiveness to mechanical and thermal stimulations has fully developed (Vos et al., 1994).

REFERENCES


Method 6: Neuropathic pain secondary to diabetic-like neuropathy induced by streptozotocin.
MATERIAL AND METHODS

Animals
Male CD1 mice aged from 6 to 8 weeks old were used in these studies. Animals were housed in groups of five, provided with food and water ad libitum and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and light in 12 h cycles (on at 07:00 h and off at 19:00 h). Experiments were carried out in a soundproof and air-regulated experimental room.

Drugs
Streptozotocin was purchased from Tocris (500mg, ref.1621) and was administered by intraperitoneal (ip) route at 200 mg/kg in a volume of 10 ml/kg. Streptozotocin was dissolved in saline immediately before administration.
Drug used for treatments was AS-19 (Ref. 1968, Tocris; Sanin et al., 2003). AS-19 was dissolved in saline. Doses were calculated based on the molecular weight of the free base and administered in a volume of 10 ml/kg through the subcutaneous (i.p) route.

_Evaluation of mechanical allodynia: manual von Frey test._
Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation, as previously reported (Chapman et al., 1994). The filament of 0.4 g and 4 g were the first and the last used, respectively. Then, the strength of the next filament was decreased when the animal responded or increased when animal did not respond. This up-down procedure was stopped four measures after the first change in animal responding and the threshold of response was calculated. Clear paw withdrawal, shaking was considered as a nociceptive-like response.

_Experimental protocol_
Because the primary indicator for the successful induction of type I diabetes using this animal model is the development of hyperglycemia, it is necessary to measure the level of glucose in the blood circulation. Diabetic hyperglycemia is defined in this study as a nonfasting plasma glucose concentration more than 250 mg/dl.
After a basal reading with von Frey hairs and one glucose level control, a dose of 200 mg/kg of streptozotocin prepared in saline was injected intraperitoneally in the 7 week after birth.
The glucose levels were monitoring one week after streptozotocin treatment to confirm the acquisition of diabetes, blood samples were collected through maxilar vein and plasma glucose levels were estimated by diagnostic Accu-Chek Sensor Comfort System kit
method. Only animals with glucose levels higher than 250 mg/dl, were selected to measure mechanical allodynia.

Readings with von Frey hairs were done 30 min after treatment with vehicle, on day 21, 24 and 29 post-streptozotocin and after treatment with AS-19 (1, 5 and 10 mg/kg) on day 22, 25 and 30 post-streptozotocin, respectively.

REFERENCES


Method 7: Carrageenan model of inflammatory pain

MATERIAL AND METHODS

Animals
Male CD1 mice aged from 6 to 8 weeks old were used in these studies. Animals were housed in groups of five, provided with food and water ad libitum and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and light in 12 h cycles (on at 07:00 h and off at 19:00 h). Experiments were carried out in a soundproof and air-regulated experimental room.

Drugs
Drugs used for treatments were: AS-19 [(2S)-(+)\)-8-(1,3,5-trimethylpyrazolin-4-yl)-2-(dimethylamino) tetralin]. AS-19 was dissolved in 1% DMSO. Doses refer to the salt weight. AS-19 was administered in a volume of 5 ml/kg through the subcutaneous (s.c.) route.

Experimental protocol
Male CD1 mice (Harlan Ibérica, Barcelona) were injected with \( \lambda \)-carrageenan (50 \( \mu \)l of a 2.5% solution) or saline (vehicle) into the plantar surface of the right hind paw. Subplantar carrageenan injection results in paw edema, allodynia and hyperalgesia, and represents a standard animal model to identify antiinflammatory and analgesic compounds. In this study, the effect of AS-19 on mechanical allodynia or thermal hyperalgesia was assessed 3 hours (acute inflammation) or 4 days (chronic inflammation) after subplantar carrageenan injection. Withdrawal threshold or latencies to mechanical or thermal stimuli were determined using the authomatic von Frey and plantar test, respectively.

**Evaluation of mechanical allodynia: authomatic von Frey test**

Mechanical allodynia was quantified by measuring the tactile threshold for hind paw withdrawal following von Frey filament stimulation. The day of the test, mice were placed into compartment enclosures in a test chamber with a framed metal mesh floor and allowed to acclimate to their environment for 1 h before testing. The mechanical stimulus was applied to the plantar surface of the right hind paw from below the mesh floor by using an automated testing device (dynamic plantar aesthesiometer; Ugo Basile, Italy) that lifts a straight filament (0.5 mm diameter). The filament exerted an increasing upward pressure on the plantar surface. The pressure increased linearly with time (0.1g/s; from 0 to 5 g in 50 s) until the mouse withdrew its paw. When the animal withdrew its hind paw, the mechanical stimulus automatically stopped and the force was recorded as the mechanical threshold. Thus, the mechanical threshold was defined as the pressure at which the sensor detected the withdrawal of the paw. A cut-off threshold of 5 g (50 s) was imposed. Paw withdrawal latencies were measured in triplicate for each animal.

**Evaluation of thermal hyperalgesia: plantar test**

Thermal hyperalgesia was assessed using the plantar test by determination of the withdrawal latencies to a thermal stimulus following a modification of the test described by Hargreaves et al. (1988). The day of the test, mice were placed in a plantar test device (Plantar test, Ugo Basile, Italy) and allowed to acclimate to their environment for 1 h before testing. The plantar test device consisted of a plastic cubicle with a glass floor through which a mobile infrared photobeam was applied onto the plantar surface of the right hind paw. Latency time to withdrawal from the thermal stimulus was determined. The intensity of the infrared photobeam was adjusted to produce mean response latencies in control untreated mice of approximately 8 to 12 seconds. The response latency was determined using a timer linked to the photodiode motion
sensors in the plantar test device. Response latency was defined as the time from the onset of exposure to the infrared photobeam to the cessation of the photobeam when the photodiode motion sensor detected the withdrawal of the paw of the mouse. Paw withdrawal latencies were measured in triplicate for each animal and the mean value was used for analysis. A cut-off latency of 20 seconds was imposed to avoid tissue damage.

REFERENCES


Method 8: Formalin model of inflammatory pain

Animals
Male CD1 mice weighted from 20 to 32 grams were used in these studies. Animals were housed in groups of five, provided with food and water ad libitum and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and light in 12 h cycles (on at 07:00 h and off at 19:00 h). Experiments were carried out in a soundproof and air-regulated experimental room.

Drugs
Drugs used for treatments were: AS-19 [(2S)-(+)\textendash8-(1,3,5-trimethylpyrazolin-4-yi)-2-(dimethylamino) tetralin]. Doses refer to the salt weight. AS-19 was dissolved in 5% Arabic gum. AS-19 was administered in a volume of 10 ml/kg through the intraperitoneal (i.p) route.

Experimental protocol
The formalin (20 μL of 5 %) was injected s.c. in the plantar surface of the right hind paw to all male CD1 mice (Harlan Ibérica, Barcelona) 5 minutes prior to receiving either AS-19 or vehicle (Arabic gum 5%). The analgesic effects of AS-19 on inhibition of each mouse’s pain behavior (licking time) were observed for a period of 15 minutes (25-40 min post-injection of the drug, phase II of the formalin test). Analgesic effect in the second phase of the formalin test has been shown to have a good correlation with analgesic efficacy on neuropathic model for both for rats and mice (Vissers et al., 2006).
REFERENCES


The examples and figures in the following section describing pharmacological trials are merely illustrative and the invention cannot be considered in any way as being restricted to these applications.

Figures:

Figure 1) Scheme summarizing experimental protocol in capsaicin test.
Mice were habituated for 120 min and received s.c. treatment with 5-HT₇ ligands or vehicle 30 min before subplantar injection of capsaicin or vehicle. Paw withdrawal latencies to a mechanical stimulus (von Frey stimulation) were determined 15 min after subplantar capsaicin or vehicle injection. To test the possible antiallodynic effect of drugs, prompt allodynic responses were induced by injecting 1μg of capsaicin followed by stimulation with 1 g of pressure. In contrast, to detect possible proalldydic effects of drugs, a low ineffective dose of 4 ng of capsaicin and a low subthreshold mechanical pressure of 0.25 g were used.

Figure 2) Effect of subplantar capsaicin injection on the paw withdrawal latency to mechanical stimulation: mechanical allodynia

Figure 2 refers to example 2a). Mice receiving subplantar injection of 1 μg capsaicin developed mechanical allodynia, as evidenced by their significantly reduced paw withdrawal latencies to stimulation with a 1 gram-pressure filament when compared to mice subplantarly injected with vehicle (1% DMSO). In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. *** p<0.001 vs. vehicle.

Figure 3) Dose-response effect of AS-19 on mechanical allodynia induced by capsaicin
Figure 3 refers to Example 2b). Mechanical allodynia in mice receiving subplantar injection of 1 µg capsaicin was dose dependently inhibited by treating mice with AS-19. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. *** p<0.001 vs. vehicle.

**Figure 4** Dose-response effect of MSD-5a on mechanical allodynia induced by capsaicin

Figure 4 refers to Example 2b). Mechanical allodynia in mice receiving subplantar injection of 1 µg capsaicin was inhibited by treating mice with MSD-5a. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. ** p<0.01; *** p<0.001 vs. vehicle.

**Figure 5** Dose-response effect of SB-258719 on mechanical allodynia induced by capsaicin

Figure 5 refers to Example 2c). Mechanical allodynia in mice receiving subplantar injection of 1 µg capsaicin was unaffected by treating mice with SB-258719 at 2.5, 5 and 10 mg/kg. In contrast, treatment with SB-258719 at 20 mg/kg significantly increased paw withdrawal latencies respect to vehicle-treated mice. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. *** p<0.001 vs vehicle.

**Figure 6** Dose-dependent reversion of the antiallodynic effect of AS-19 by SB-258719 in the capsaicin test.

Figure 6 refers to Example 2c). Mechanical allodynia in mice receiving subplantar injection of 1 µg capsaicin was unaffected by treating mice with SB-258719 at 2.5 and 5 mg/kg, but these doses of SB-258719 were able to reverse the antiallodynic effect of 5 mg/kg of AS-19. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. ** p<0.01 vs. vehicle; # p<0.05 vs. AS-19 (5).

**Figure 7** Reversion of the antiallodynic effect of AS-19 by SB-269970 in the capsaicin test.

Figure 7 refers to Example 2c). Mechanical allodynia in mice receiving subplantar injection of 1 µg capsaicin was unaffected by the 5-HT7 antagonist SB-269970 (10 mg/kg) but, when co-administered with AS-19 (5 mg/kg), it blocked the antiallodynic effect of the agonist. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. ** p<0.01 vs. vehicle; ### p<0.001 vs. AS-19 (5).
Figure 8) Reversion of the antiallodynic effect of MSD-5a by SB-258719 in the capsaicin test.

Figure 8 refers to Example 2c). Mechanical allodynia in mice receiving subplantar injection of 1 μg capsaicin was inhibited by MSD-5a (10 mg/kg) and SB-258719 (5 mg/kg) blocked this antiallodynic effect. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. *** p<0.001 vs. vehicle; # # # p<0.001 vs. MSD-5a (10).

Figure 9) Dose-response effect of WAY-100535 on mechanical allodynia induced by capsaicin

Figure 9 refers to Example 2c). Mechanical allodynia in mice receiving subplantar injection of 1 μg capsaicin was unaffected by treating mice with WAY-100635 at 0.1 and 0.3 mg/kg, but not at 0.5 and 1 mg/kg, doses at which a significant increase in paw withdrawal latency was observed. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. * p<0.05 vs vehicle.

Figure 10) Reversion of the antiallodynic effect of F-13640 by WAY-100635 in the capsaicin test.

Figure 10 refers to Example 2c). Mechanical allodynia in mice receiving subplantar injection of 1 μg capsaicin was inhibited by F-13640 (1 mg/kg) and WAY-100635 (0.3 mg/kg) blocked this antiallodynic effect. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. ** p<0.01 vs. vehicle; # # # p<0.001 vs. F-13640 (1).

Figure 11) Reversion of the antiallodynic effect of AS-19 by SB-258719 or WAY-100635 in the capsaicin test.

Figure 11 refers to Example 2c). Mechanical allodynia in mice receiving subplantar injection of 1 μg capsaicin was unaffected after treatment with SB-258719 (5 mg/kg) or WAY-100635 (0.3 mg/kg). However, SB-258719 but not WAY-100635 significantly inhibited the antiallodynic effect of AS-19. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. * p<0.05; *** p<0.001 vs. vehicle; # # p<0.01 vs. AS-19 (10).

Figure 12) Potentiation of capsaicin-induced mechanical allodynia by SB-258719 and SB-269970.
Figure 12 refers to Example 2d). In the absence of capsaicin or when a minor dose of 4 ng was injected into the paw, mice did not exhibit allodynia when mechanically stimulated with a low upward pressure of 0.25 g. However, allodynia was evidenced in mice receiving 4 ng of capsaicin when treated with the 5-HT\textsubscript{7} antagonists SB-258719 or SB-269970 at 10 mg/Kg, showing a proalldynic effect of 5-HT\textsubscript{7} receptor antagonists. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. *** p<0.001 vs. "without capsaicin".

**Figure 13** Dose-dependent potentiation of capsaicin-induced mechanical allodynia by SB-258719.

Figure 13 refers to Example 2d). SB-258719 dose-dependently promoted allodynia in mice subplantarly injected with a minor dose of 4 ng and mechanically stimulated with a low upward pressure of 0.25 g. In addition to the graph summarizing the results, the figure shows the scheme of the experimental protocol. *** p<0.001 vs. vehicle.

**Figure 14** Dose-response reversion of the proalldynic effect of SB-258719 and SB-269970 by AS-19 in the capsaicin test.

Figure 14 refers to Example 2d). Subcutaneous treatment with AS-19 had no effect in non-alldynic conditions after subplantar injection of 4 ng of capsaicin and low pressure (0.25 g) stimulation, but it was able to reverse the proalldynic effect of SB-258719 or SB-269970 (5 mg/Kg, s.c.). ** p<0.01, *** p<0.001 vs. vehicle; \# p<0.05, ## p<0.01 vs. SB-258719 (5); && p<0.01 vs. SB-269970 (5).

**Figure 15** Scheme summarizing experimental protocol in partial sciatic nerve ligation: Latin square design.

Mice were habituated during 4 days and basal pre-injury values were obtained during the next 2 consecutive days by means of the automatic von Frey or the plantar test. The following day mice were subjected to surgery and 5 days later post-injury baseline values were obtained in the mentioned tests. On day 10 post-injury mice received vehicle and measurements were done to obtain pre-treatment basal values. Measurements in drug-treated mice were done on days 11-13 post-injury following a Latin square design. Finally, post-treatment basal values were obtained on day 14. Habituation of mice during the first 4 days and every time before measurements lasted 1 h. When treated, measurements were always done 30 min after administration of the vehicle or the tested compound.
**Figure 16.** Scheme summarizing experimental protocol in partial sciatic nerve ligation: Single dose design. Mice were habituated during 1-2 days and basal pre-injury values were obtained during the next day by means of the manual von Frey or the plantar test. The following day mice were subjected to surgery and 5 days later post-injury baseline values were obtained in the mentioned tests. On day 10 post-injury mice received vehicle and measurements were done to obtain pre-treatment basal values. Measurements in drug-treated mice were done on day 11 post-injury. Habituation of mice during the first 1-2 days and every time before measurements lasted 2 h. When treated, manual von Frey and plantar test were done 30 and 45 min, respectively, after administration of the vehicle or the tested compound.

**Figure 17.** Dose-response effect of AS-19 on mechanical allodynia induced by partial sciatic nerve ligation.

Figure 17 refers to example 3a). Pressure threshold for evoking ipsilateral hind paw withdrawal to von Frey filament stimulation was evaluated in nerve-injured (A) and sham-operated (B) mice before surgery (pre-injury), on day 10 post-injury after treatment with vehicle (pre-treatment), on days 11-13 post-injury after treatment with different doses of AS-19 and finally on day 14 post-injury after treatment with vehicle (post-treatment). Note that mechanical allodynia develops after partial sciatic nerve ligation (but not after sham operation) and that AS-19 at doses of 1-10 mg/kg exerted antiallodynic effects. *** p<0.001 vs Pre-Injury; # p<0.05, ## p<0.01, ### p<0.001 vs Pre- and Post-Treatment (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 18.** Dose-response effect of AS-19 on thermal hyperalgesia induced by partial sciatic nerve ligation.

Figure 18 refers to example 3b). Latency of ipsilateral hind paw withdrawal to thermal stimulus was evaluated in nerve-injured (A) and sham-operated (B) mice before surgery (pre-injury), on day 10 post-injury after treatment with vehicle (pre-treatment), on days 11-13 post-injury after treatment with different doses of AS-19 and finally on day 14 post-injury after treatment with vehicle (post-treatment). Note that thermal hyperalgesia develops after partial sciatic nerve ligation (but not after sham operation) and that AS-19 at doses of 1 and 10 mg/kg exerted antihyperalgesic effects. *** p<0.01 vs Pre-Injury; # p<0.05 vs Pre- and Post-Treatment (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 19.** Dose-response effect of MSD-5a on mechanical allodynia induced by partial sciatic nerve ligation.
Figure 19 refers to example 3a). Pressure threshold for evoking ipsilateral hind paw withdrawal to von Frey filament stimulation was evaluated in nerve-injured (A) and sham-operated (B) mice before surgery (pre-injury), on day 10 post-injury after treatment with vehicle (pre-treatment), on days 11-13 post-injury after treatment with different doses of MSD-5a and finally on day 14 post-injury after treatment with vehicle (post-treatment). Note that mechanical allodynia develops after partial sciatic nerve ligation (but not after sham operation) and that MSD-5a at doses of 1-3 mg/kg exerted antiallodynic effects. * p<0.05, *** p<0.001 vs Pre-Injury; ### p<0.001 vs Pre-Treatment (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 20** Dose-response effect of MSD-5a on thermal hyperalgesia induced by partial sciatic nerve ligation.

Figure 20 refers to example 3b). Latency of ipsilateral hind paw withdrawal to thermal stimulus was evaluated in nerve-injured (A) and sham-operated (B) mice before surgery (pre-injury), on day 10 post-injury after treatment with vehicle (pre-treatment), on days 11-13 post-injury after treatment with different doses of MSD-5a and finally on day 14 post-injury after treatment with vehicle (post-treatment). Note that thermal hyperalgesia develops after partial sciatic nerve ligation (but not after sham operation) and that MSD-5a at doses of 1-3 mg/kg exerted antihyperalgesic effects. ** p<0.01 vs Pre-Injury; *** p<0.01 ### p<0.001 vs Pre- and Post-Treatment (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 21** Dose-response proalldynic effect of SB-258719 on mechanical allodynia induced by partial sciatic nerve ligation.

Figure 21 refers to example 3a). Pressure threshold for evoking ipsilateral hind paw withdrawal to von Frey filament stimulation was evaluated in nerve-injured (A) and sham-operated (B) mice before surgery (pre-injury), on day 10 post-injury after treatment with vehicle (pre-treatment), on days 11-13 post-injury after treatment with different doses of SB-258719 and finally on day 14 post-injury after treatment with vehicle (post-treatment). Note that mechanical allodynia develops after partial sciatic nerve ligation (but not after sham operation) and that SB-258719 at doses of 10 mg/kg exerted proalldynic effects in neuropathic mice, but also in sham mice. * p<0.05, *** p<0.001 vs Pre-Injury; # p<0.05 vs Pre-Treatment (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 22** No effect of SB-258719 on thermal hyperalgesia induced by partial sciatic nerve ligation.
Figure 22 refers to example 3b). Latency of ipsilateral hind paw withdrawal to thermal stimulus was evaluated in nerve-injured (A) and sham-operated (B) mice before surgery (pre-injury), on day 10 post-injury after treatment with vehicle (pre-treatment), on days 11-13 post-injury after treatment with different doses of SB-258719 and finally on day 14 post-injury after treatment with vehicle (post-treatment). Note that thermal hyperalgesia develops after partial sciatic nerve ligation (but not after sham operation) and that SB-258719 at doses of 0.1-10 mg/kg did not exert any effects. ** p<0.01, *** p<0.001 vs Pre-Injury; (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 23** Reversion of the effects of AS-19 and MSD-5a by SB-258719 on mechanical allodynia induced by partial sciatic nerve ligation.

Figure 23 refers to example 3a). Pressure threshold for evoking ipsilateral hind paw withdrawal to von Frey filament stimulation was evaluated in nerve-injured mice before surgery (pre-injury), on day 10 post-injury after treatment with vehicle (pre-treatment), on day 11 post-injury after treatment with an agonist, an antagonist or a combination of both. Note that SB-258719 (5 mg/Kg) blocked the antiallodynic effects exerted by AS-19 (5 mg/kg) and MSD-5a (10 mg/kg) in nerve-injured mice. *** p<0.001 vs Pre-Injury; ** # # p<0.001 vs Pre-Treatment; & & p<0.01 vs MSD-5a (10); &&& p<0.001 vs AS-19 (5); (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 24** Reversion of the effects of AS-19 and MSD-5a by SB-258719 on thermal hyperalgesia induced by partial sciatic nerve ligation.

Figure 24 refers to example 3b). Latency of ipsilateral hind paw withdrawal to thermal stimulus was evaluated in nerve-injured mice before surgery (pre-injury), on day 10 post-injury after treatment with vehicle (pre-treatment), on day 11 post-injury after treatment with an agonist, an antagonist or a combination of both. Note that SB-258719 (5 mg/Kg) blocked significantly the antihyperalgesic effects exerted by AS-19 (5 mg/kg) and had a tendency to revert MSD-5a (10 mg/kg) without significance in nerve-injured mice. * p<0.05, ** p<0.01, *** p<0.001 vs Pre-Injury; # p<0.05, # # p<0.01 vs Pre-Treatment (Newman-Keuls Multiple Comparison Test post-ANOVA); & p<0.05 vs AS-19 (5) (Unpaired student's t-test).

**Figure 25** Anti-hyperalgesic / anti-allodynic effects of 5-HT7 receptor stimulation by AS 19 in sciatic nerve-ligated rats

**Figure 26** Anti-hyperalgesic / anti-allodynic effects of 5-HT7 receptor stimulation by 5a-MSD in sciatic nerve-ligated rats.
Figure 27) Blockade by SB-269970 of AS 19-induced anti-hyperalgesia / anti-allodynia in sciatic nerve-ligated rats.

Figure 28) Blockade by SB-258719 of 5a-MSD-induced anti-hyperalgesia / anti-allodynia in sciatic nerve-ligated rats.

Figure 29) Anti-hyperalgesic / anti-allodynic effects of 5-HT7 receptor stimulation by AS 19 in infraorbital nerve-ligated rats.

Figure 30) Anti-hyperalgesic / anti-allodynic effects of 5-HT7 receptor stimulation by 5a-MSD in infraorbital nerve-ligated rats.

Figure 31) Blockade by SB 269970 of AS 19-induced anti-hyperalgesia / anti-allodynia in infraorbital nerve-ligated rats.

Figure 32) Blockade by SB 269970 of 5a-MSD-induced anti-hyperalgesia / anti-allodynia in infraorbital nerve-ligated rats.

Figure 33) Dose-response effect of AS-19 on mechanical allodynia on neuropathic pain secondary to diabetic-like neuropathy induced by streptozotocin.
Pressure threshold for evoking ipsilateral hind paw withdrawal to von Frey filament stimulation was evaluated in diabetic mice before treatment with streptozotocin (basal), on day 21, 24 and 29 post-streptozotocin after treatment with vehicle, on day 22, 25 and 30 post-streptozotocin after treatment with AS-19 (1, 5 and 10 mg/kg), a 5-HT7 agonist. AS-19 at 10 mg/kg exerted a clear antiallodynic effect in diabetic mice. ** p<0.001 vs Basal (Newman-Keuls Multiple Comparison Test post-ANOVA); ## p<0.01 vs Vehicle (paired student's t-test).

Figure 34) Effect of AS-19 treatment on carrageenan-induced mechanical allodynia (acute inflammation, 3 hours)
Three hours after carrageenan injection, mechanical allodynia was observed in the affected ipsilateral (right) paw when comparing with the control contralateral (left) paw. Administration of A-19 (10 mg/Kg, s.c.) produces a significant antiallodynic effect. Data are mean ± SEM of 10 mice per group. * p<0.05, *** p<0.001 vs. contralateral
paw of the vehicle treated group; ### p<0.001 vs. ipsilateral paw of the vehicle treated group (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 35** Effect of AS-19 treatment on carrageenan-induced thermal hyperalgesia (chronic inflammation, 4 days)

Four days after carrageenan injection, thermal hyperalgesia was apparent in the affected ipsilateral paw of vehicle-treated mice. Treatment with AS-19 (10 mg/Kg, s.c.) abolished thermal hyperalgesia. Data are mean ± SEM of 10 mice per group. ** p<0.01 vs. contralateral paw of the vehicle treated group; ## p<0.01 vs. ipsilateral paw of the vehicle treated group (Newman-Keuls Multiple Comparison Test post-ANOVA).

**Figure 36** Effect of AS-19 treatment on formalin-induced inflammatory pain

Formalin injection induces inflammatory pain as evidenced by increased paw licking time in the vehicle treated group. Treatment with AS-19 (5 mg/Kg, i.p.) reduces the duration of the paw licking induced by formalin. Data are mean ± SEM of 8 mice per group. *** p<0.001 vs. vehicle treated group (Newman-Keuls Multiple Comparison Test post-ANOVA).

Examples:

**Example 1: Binding and functionality**

**Selectivity and functionality of drugs**

There is a remarkable need of selective 5-HT7 ligands, particularly agonists. This has hindered pharmacological studies exploring the role played by this receptor. In this study, pharmacological evidence is presented to prove the involvement of the 5-HT7 receptor in the control of nociceptive behaviors. Information on the selectivity of the pharmacological tools used is thus a key issue and has been compiled in this section. AS-19 is a 5-HT7 receptor agonist (Ki = 0.605 nM, Emax = 77.0±2.3 %, EC50 = 8.87±0.57 nM). Unfortunately, as pointed up by Perez-Garcia and Meneses (2005), there is a lack of information in the literature regarding its affinity for receptors other than 5-HT7. Hence, the affinity of AS-19 for a total of 70 receptors, transporters or ion channels was evaluated in a commercial screening package (MDS Pharma Services) and the binding profile was presented in Table 1. Not only AS-19 has high affinity for 5-HT7 receptors but also has significant affinity for the 5-HT1A (Ki = 89.7 nM), and 5-HT5A (Ki = 98.5 nM) receptors, showing no significant affinity for other 5-HT receptor
subtypes or other receptors, transporters or ion channels included in the screening package.

MSD-5a is also a 5-HT₇ agonist (Ki = 0.6 nM; Emax = 60.8±1.9 %; EC50 = 6.82±1.45 nM), but its selectivity against 5-HT₁A (Ki = 16 nM) is not as high as in the case of AS-19 (Table 1). In addition, it has partial agonist activity, giving 80% of the response of the full agonist 5-CT in 5-HT₇ receptors (Thomson et al., 2004).

SB-258719 was identified as the first selective 5-HT₇ receptor antagonist, with 100-fold selectivity over a range of other receptors (Forbes et al., 1998). SB-269970 was also identified as a potent and selective 5-HT₇ receptor antagonist (Lovell et al., 2000). Particularly noteworthy is the lack of affinity at the 5-HT₁A receptor by both 5-HT₇ receptor antagonists used in this study (Forbes et al., 1998; Lovell et al., 2000). Regarding functionality, SB-258719 and SB-269970 have been described as a pure antagonist and an inverse agonist, respectively (Mahé et al., 2004). However, both 5-HT₇ antagonists should be considered as inverse agonists as negative intrinsic activity for these compounds has been demonstrated (Romero et al., 2006).

The doses chosen in our experiments of either agonist or antagonists have already been tested in other in vivo experiments like assays sensitive to memory formation, potential psychotic and anxiety status or antidepressant drugs (Perez-García et al., 2005; Guscott et al., 2005). The knowledge of these compounds has helped us to choose the best range of doses to study in order to avoid finding side effects.
Table 1. Receptor affinity profiles in radioligand binding assays

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Affinity [K_i (nM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AS-19</td>
</tr>
<tr>
<td>h5-HT_{1A}</td>
<td>89.7</td>
</tr>
<tr>
<td>h5-HT_{1B}</td>
<td>--</td>
</tr>
<tr>
<td>h5-HT_{1D}</td>
<td>--</td>
</tr>
<tr>
<td>h5-HT_{1E}</td>
<td>--</td>
</tr>
<tr>
<td>h5-HT_{1F}</td>
<td>--</td>
</tr>
<tr>
<td>r5-HT_{1} non-selective</td>
<td>48.1</td>
</tr>
<tr>
<td>h5-HT_{2A}</td>
<td>--</td>
</tr>
<tr>
<td>h5-HT_{2B}</td>
<td>n.s.</td>
</tr>
<tr>
<td>h5-HT_{2C}</td>
<td>n.s.</td>
</tr>
<tr>
<td>r5-HT_{2} non-selective</td>
<td>n.s.</td>
</tr>
<tr>
<td>h5-HT_{3}</td>
<td>n.s.</td>
</tr>
<tr>
<td>h5-HT_{4}</td>
<td>--</td>
</tr>
<tr>
<td>gp5-HT_{4}</td>
<td>n.s.</td>
</tr>
<tr>
<td>h5-HT_{5A}</td>
<td>98.5</td>
</tr>
<tr>
<td>h5-HT_{6}</td>
<td>n.s.</td>
</tr>
<tr>
<td>h5-HT_{7}</td>
<td>0.6</td>
</tr>
</tbody>
</table>

n.s. not significant (K_i > 1 μM or % inhibition at 1 μM lower than 50%); -- data not available.

h, human; gp, guinea pig; m, mouse; r, rat; rb, rabbit. Data MDS Pharma Services, Cerep, Thomson et al. (2004).

Table 2. Functionality profiles.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Emax (%)</th>
<th>EC50 (nM)</th>
<th>pEC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>100.0</td>
<td>11.4 ± 0.58</td>
<td>7.89 ± 0.02</td>
</tr>
<tr>
<td>MSD-5a</td>
<td>60.8 ± 1.9</td>
<td>6.82 ± 1.45</td>
<td>8.21 ± 0.13</td>
</tr>
<tr>
<td>AS-19</td>
<td>77.0 ± 2.3</td>
<td>8.87 ± 0.57</td>
<td>8.06 ± 0.03</td>
</tr>
</tbody>
</table>

Emax and EC50 values shown in the table were obtained from cAMP-mediated concentration-response curves.

Data correspond to mean ± SEM values of at least three independent experiments performed in duplicate.
Example 2: Capsaicin test:

a) Capsaicin sensitization.
Capsaicin sensitization produces mechanical allodynia. Mice injected with capsaicin (1μg) into the mid-plantar surface of the right hind paw developed mechanical allodynia 15 min after the injection. The pressure exerted by the filament (1 g) was below the threshold of feeling of paws from control mice (control withdrawal latencies of mice injected with 1% DMSO were close to the 60 sec cut-off). However, 15 min after subplantar capsaicin injection, the same “innocuous”, “non-noxious” pressure (1 g) was perceived as painful and the withdrawal latency was severely reduced (Fig. 2). This abnormal pain sensitivity to previously non-painful mechanical stimuli is known as mechanical allodynia and in our model results from capsaicin sensitization.

b) Selective 5-HT7 receptor agonists dose-dependently inhibit capsaicin-induced mechanical allodynia
Systemic subcutaneous administration of the 5-HT7 receptor agonist AS-19 dose-dependently inhibited capsaicin-induced mechanical allodynia (Fig. 3). Significantly increased paw withdrawal latencies of mice treated with AS-19 were found at the doses of 5, 10 and 20 mg/Kg when comparing to mice treated with vehicle. The antiallodynic effect reached the maximum at the dose of 10 mg/kg.

Similar to AS-19, MSD-5a, another 5-HT7 receptor agonist used in this study, exerted antiallodynic effects (Fig. 4). At doses of 3, 10 and 30 mg/kg, administration of MSD-5a to mice receiving capsaicin increased the latencies of nociceptive paw withdrawal responses to mechanical stimulation compared to vehicle-treated mice. Differences were significant (p>0.05) at all three tested doses and reached the maximum at 10 mg/kg.

c) Reversion of the antiallodynic effect of 5-HT7 receptor agonists by selective 5-HT7 receptor antagonists
In order to confirm that activation of 5-HT7 receptors is unambiguously responsible for the inhibition of capsaicin-induced mechanical allodynia exerted by AS-19 and MSD-5a, we used two different 5-HT7 receptor antagonists (SB-258719 and SB-269970) to try to pharmacologically reverse the antiallodynic effect of agonists.
First we performed a dose-response study of the effect of SB-258719 by itself on capsaicin-induced mechanical allodynia (Fig. 5). SB-258719 was s.c injected at doses of 2.5, 5, 10 and 20 mg/kg and no effects were observed except at the highest dose, at which an antiallodynic effect was evidenced.
In the next experiment, low doses of SB-258719 having no antiallodynic effects were chosen to try to reverse the antiallodynic effect exerted by 5 mg/kg of AS-19. As shown in Figure 6, SB-258719 at 2.5 and 5 mg/kg had no effect whereas treatment with AS-19 (5 mg/kg) increased paw withdrawal latencies. Interestingly, the analgesic (antiallodynic) effect of AS-19 was dose-dependently inhibited when co-administered with SB-258719.

Pharmacological reversion of the antiallodynic effect of AS-19 was also successfully accomplished by using another 5HT7 receptor antagonist, SB-269970, at a dose (10 mg/kg) that by itself did not modify paw withdrawal latencies after subplantar capsaicin injection (Fig. 7). Finally, we also blocked the antiallodynic effect produced by the other 5HT7 receptor agonist, MSD-5a (10 mg/kg), using the antagonist SB-258719 (5 mg/kg) (Fig. 8) Altogether, preceding data using two different 5HT7 receptor agonists and confirmation by pharmacological reversion with two different 5HT7 receptor antagonists, strongly suggest that activation of 5HT7 receptors exerts an antiallodynic effect after capsaicin sensitization.

As AS-19 has some affinity for 5-HT1A receptors in addition to binding to 5HT7 receptors (see Table 1) and as 5-HT1A receptor agonists are known to exert analgesic effects in a number of animal models (Colpaert, 2006), we next evaluated the possibility that actions on 5-HT1A receptors could also contribute to the antiallodynic effects of AS-19. To accomplish that, AS-19 was administered together with the selective 5-HT1A antagonist WAY-100635 (Foster et al., 1995; Khawaja et al., 1995) at 0.3 mg/kg, a dose that had no effect on capsaicin-induced mechanical allodynia (Fig. 9) but largely blocks the effect of the selective 5-HT1A agonist F-13640 (Colpaert et al., 2002) (Fig. 10). Similarly, this dose has been shown to block the 5-HT1A-mediated effects induced by 8-OH-DPAT (ID50 = 0.01 mg/kg) (Foster et al., 1995) and other 5-HT1A agonists (Reissig et al., 2005; You et al., 2005). Intriguingly, the antiallodynic effect of AS-19 at 5 and 10 mg/kg was unaffected in the presence of WAY-100635 but was abolished by SB-258719 (Fig. 11), indicating that activation of 5-HT7 but not of 5-HT1A receptors underlies the antiallodynic effect exerted by AS-19.

d) Selective 5-HT7 receptor antagonists exert proalldyonic effects

Activation of 5-HT7 receptors by selective agonists has an antiallodynic effect in mice sensitized with capsaicin, but do selective 5-HT7 receptor antagonists exert a proalldyonic effect? To investigate this possibility we reduced both the dose of capsaicin (from 1 μg to 4 ng) and the upward pressure exerted by the filament (from 1 g to 0.25 g). Under these conditions, mice did not display mechanical allodynia. However, when treated with SB-258719 or SB-269970, mice receiving this minor
amount of capsaicin (4 ng) and subjected to this low-pressure stimulation (0.25 g) showed a marked allodynia (Fig. 12). Interestingly, the proalldynic effect of 5-HT7 receptor antagonists was observed in mice injected with 4 ng of capsaicin but not in mice injected with vehicle (1% DMSO), suggesting that a minimal sensitizing challenge is needed for the antagonists to exert their effect.

The proalldynic effect of SB-258719 was dose-dependent (Fig. 13) and was inhibited by AS-19 dose dependently (Fig. 14). Promotion of alldynia by the other antagonist, SB-269970, was also inhibited by AS-19 (Fig. 14), pointing to the involvement of 5-HT7 receptors in such an effect.

CONCLUSIONS

5-HT7 receptors play a key role in the control of mechanical alldynia elicited by capsaicin sensitization:

- **Activation of 5HT7 receptors by selective agonists dose-dependently inhibits capsaicin-induced mechanical alldynia.**
  
  The antialldynic effect was evidenced by using two different selective 5HT7 receptor agonists (AS-19 and MSD-5a). AS-19 and MSD-5a blocked this behavioral manifestation of pain and restored the nociceptive response of capsaicin-sensitized mice to baseline values found in control unaffected mice.

- **In contrast, blocking of 5HT7 receptors by selective antagonists produces a dose-dependent proalldynic effect.**
  
  The proalldynic effect was evidenced by using two different selective 5HT7 receptor antagonists (SB-258719 and SB-269970).

- **Antialldynic effects by agonists and proalldynic effects by antagonists can be reversed by antagonists and agonists, respectively.**
  
  The analgesic (antialldynic) effects of AS-19 and MSD-5a were both inhibited by using two different selective 5HT7 receptor antagonists (SB-258719 and SB-269970). In the same way, promotion of alldynia by the antagonists SB-258719 and SB-269970 was blocked by the AS-19 agonist.

These results reveal the involvement of the 5-HT7 receptor in the control of pain in conditions involving central sensitization and point to a new potential
therapeutic use of 5-HT_{7} receptor agonists as analgesics, especially in neuropathic pain.

**Example 3: Partial sciatic nerve ligation**

Neuropathic pain refers to pain as a result of damage (due to injury or disease) to the nervous system including nerves, spinal cord and certain CNS regions (Woolf and Mannion, 1999; Zimmermann, 2001). Patients with neuropathic pain often suffer from spontaneous pain, allodynia (pain response to normally innocuous stimuli) and hyperalgesia (exaggerated pain evoked by noxious stimuli). Direct evaluation of spontaneous pain in animals is not possible, although some signs such as excessive grooming, changes in exploratory behavior or weight bearing have been suggested as indications of spontaneous pain. In contrast, evoked pain (allodynia and hyperalgesia) to thermal or mechanical stimuli can be clearly observed in most animal models of neuropathic pain. From the mechanistic point of view, neuropathic pain-associated behaviors, allodynia and hyperalgesia, revolve around the notion of central sensitization, whereby injury-evoked increased and persistent afferent discharges cause spinal cord neurons to reduce their stimulation threshold, to expand their receptive fields and to increase their responsiveness to different peripheral drives (Woolf, 1983; Hylden et al., 1989; Woolf and Thompson 1991; Coderre and Katz, 1997).

Most neuropathic pain models are made by producing diseases or causing injuries to peripheral nerves. These methods to induce neuropathic pain differ in the location and type of peripheral nerve injury. The latter includes transection, loose or tight ligation, crush, perineural inflammation, tumor invasion, infection and toxic affectation (Wang and Wang, 2003). In an attempt to simulate causalgia as a result of partial nerve injury in humans, Seltzer et al. (1990) developed a rat model of neuropathic pain involving the ligation of 1/3 to 1/2 of the thickness of the sciatic nerve. Partial sciatic nerve ligated rats exhibit marked allodynia to von Frey hair stimulation and hyperalgesia to both thermal and mechanical noxious stimuli in the ipsilateral nerve-ligated paw. The evoked pain appears within hours after ligation and last for over 7 months. Similar results were obtained in mice by Malmberg and Basbaum (1998), who established some additional behavioral and neuroanatomical correlates and pointed up the reliability and objectivity of the partial sciatic nerve ligation model of neuropathic pain in mouse.

**a) Effect of 5-HT_{7} receptor ligands in neuropathic pain: von Frey test - mechanical allodynia**
Partial sciatic nerve ligation induced mechanical allodynia. This was evidenced as a reduced pressure threshold for evoking withdrawal of the ipsilateral hind paw on day 10 post-injury compared to baseline pre-injury values (Fig. 17A). Systemic administration of the 5-HT₁ receptor agonist AS-19 on days 11-13 at doses of 1, 5 and 10 mg/kg significantly inhibited mechanical allodynia. At all these three doses, AS-19 restored thresholds of the nociceptive withdrawal response of nerve-injured paws to baseline pre-injury values. The analgesic (antiallodynic) effect disappeared as soon as AS-19 treatment was stopped (post-treatment thresholds the day after administration were indistinguishable from pre-treatment ones). Treatments with lower doses (0.1 and 0.3 mg/kg) of AS-19 were ineffective as no modification of this behavioral manifestation of neuropathic pain was found compared to vehicle-treated mice.

Sham operation did not induce mechanical allodynia but slight (not significant) reductions of the threshold of the nociceptive withdrawal response were usually found in sham-operated mice 10 days after surgery (Fig. 17B). AS-19 only produced minor effects in sham-operated mice although at doses of 1 and 5 mg/kg AS-19 increased the nociceptive threshold.

Similar to AS-19, MSD-5a, another 5-HT₁ receptor agonist used in this study, exerted antiallodynic effects (Fig. 19). Systemic administration of MSD-5a on days 11-13 at doses of 1 and 3 mg/kg significantly inhibited mechanical allodynia. At these doses, MSD-5a restored thresholds of the nociceptive withdrawal response of nerve-injured paws to baseline pre-injury values. The analgesic (antiallodynic) effect disappeared the following day after last administration of MSD-5a (threshold were indistinguishable from pre-treatment one). Treatment with lower dose (0.3 mg/kg) of MSD-5a was ineffective as no modification of this behavioral manifestation of neuropathic pain was found compared to vehicle-treated mice.

Sham operation did not induce mechanical allodynia but slight (not significant) reductions of the threshold of the nociceptive withdrawal response were usually found in sham-operated mice 14 days after surgery (Fig. 19B). MSD-5a did not produce any effects in sham-operated mice.

Activation of 5-HT₁ receptors by the selective agonist AS-19 has antiallodynic effects in nerve-injured mice, but to investigate the possibility that a selective 5-HT₁ receptor antagonist exerts proalldyic effects, the 5-HT₁ receptor antagonist SB-258719 was administered to neuropathic mice. We observed that treatment with SB-258719 at 10 mg/kg significantly decreased the mechanical threshold for evoking withdrawal of the ipsilateral, nerve-injured hind paw below post-injury values (Fig. 21A). Interestingly,
treatment with SB-258719 not only promoted allodynia in nerve-injured mice but it was also able to induce mechanical allodynia in sham-operated mice (Fig. 21B). In order to strengthen the notion that activation of 5-HT7 receptors is responsible for the analgesic effect exerted by AS-19 and MSD-5a it was tried to pharmacologically reverse its antiallodynic effect with the antagonist SB-258719. The antagonist SB-258719 at the dose of 5 mg/Kg had no antiallodynic effect and was able to reverse the antiallodynic effect exerted by AS-19 and MSD-5a at 5 and 10 mg/kg, respectively (Fig. 23).

b) Effect of 5-HT7 receptor ligands in neuropathic pain: plantar test - thermal hyperalgesia

Partial sciatic nerve ligature induced thermal hyperalgesia as evidenced by decreased paw withdrawal latency to thermal stimulus on day 10 post-injury compared to baseline pre-injury values (Fig. 18A). However, a significant decrease of such a hyperalgesia was observed on days 11-13 post-injury in mice receiving treatment with the 5-HT7 receptor agonist AS-19. In fact, treatment with AS-19 at doses of 1 and 10 mg/kg increased the withdrawal latency of nerve-injured paws to levels indistinguishable from control baseline pre-injury values. Lower doses (0.1 mg/kg) of AS-19 did not exert analgesic (antihyperalgesic) effect. Sham operation did not produce any significant modification of the nociceptive response and administration of AS-19 to sham-operated mice had no effects (Fig. 18B).

Similar to AS-19, MSD-5a, another 5-HT7 receptor agonist used in this study, at doses of 1 and 3 mg/kg increased the withdrawal latency of nerve-injured paws to levels indistinguishable from control baseline pre-injury values (Fig. 20A). Lower doses (0.3 mg/kg) of MSD-5a did not exert analgesic (antihyperalgesic) effect. Sham operation did not produce any significant modification of the nociceptive response and administration of MSD-5a to sham-operated mice had no effects (Fig. 20B).

Interestingly, treatment with SB-258719 did not promote hyperalgesia in nerve-injured mice (Fig. 22A) but it was able to reverse the antihyperalgesic effect of 5-HT7 agonists (Fig 24). As shown in Fig. 24, the antihyperalgesic effect of the agonists, AS-19 (5 mg/kg) and MSD-5a (10 mg/kg) was inhibited when co-administered with the antagonist SB-258719 (5 mg/kg).

CONCLUSIONS
5-HT₇ receptors play a key role in the control of neuropathic pain:

- Activation of 5HT₇ receptors exerts an analgesic effect in neuropathic pain: the 5HT₇ receptor agonists, AS-19 and MSD-5a inhibits neuropathic pain-associated behaviors (mechanical allodynia and thermal hyperalgesia).

  The antiallodynic and antihyperalgesic effects were fully achieved by using low doses (1 mg/kg) of the selective 5HT₇ receptor agonist AS-19.

- In contrast, blocking of 5HT₇ receptors promotes neuropathic pain: the 5HT₇ receptor antagonist SB-258719 exacerbates the behavioral manifestations of neuropathic pain (mechanical allodynia and thermal hyperalgesia).

  The selective 5HT₇ receptor antagonist SB-258719 enhances allodynia and hyperalgesia in neuropathic pain conditions after nerve injury. In addition, SB-258719 precipitated the appearance behavioral manifestations of neuropathic pain in sham-operated mice.

- Analgesic effect by the 5HT₇ receptor agonist and algiesic effect by the 5HT₇ receptor antagonist can be pharmacologically reversed by co-administering both compounds

These results reveal the involvement of the 5-HT₇ receptor in the control of neuropathic pain and point to a new potential therapeutic use of 5-HT₇ receptor agonists as analgesics.

**Example 4: Chronic constriction injury of the sciatic nerve (CCI-SN)**

Direct 5-HT₇ receptor stimulation by AS-19 and 5a-MSD exerts clear-cut antihyperalgesic effects in sciatic nerve-ligatured rats (Fig. 25 and 26, respectively). These effects are blocked by 5-HT₇ antagonists (Fig. 27 and 28).

Direct 5-HT₇ receptor stimulation by AS-19 exerts long lasting antihyperalgesic effects in sciatic nerve-ligatured rats (Fig. 25 and 26).
**Example 5: Chronic constriction injury of the infraorbital nerve (CCI-ION)**

Following the operation the response threshold (g) is reduced considerably in the nerve-injured rats and saline treatment produced no modification of the nociceptive threshold (Fig. 29-32). On the other hand, 5-HT$_7$ agonists AS-19 and 5a-MSD clearly increased the threshold (Fig. 29 and 30). The antiallodynic effect of 5-HT$_7$ agonist is blocked by the 5-HT$_7$ antagonist (Fig. 31 and 32).

AS-19 is quite potent and very effective to reduce mechanical allodynia in infraorbital nerve-ligated rats.

**CONCLUSIONS:**

- 5-HT$_7$ receptors play a key role in the control of neuropathic pain in rats.
- 5-HT$_7$ receptors may be a relevant target for alleviating both cephalic and extracephalic (somatic) neuropathic pain.

**Example 6: Neuropathic pain secondary to diabetic-like neuropathy induced by streptozotocin.**

Neuropathy is one of the most common complications of diabetes mellitus, a serious chronic disease. An estimated 50% of diabetics develop some form of peripheral neuropathy, with approximately 32% suffering from chronic, severe, and unremitting pain. The most frequently described manifestation of diabetic neuropathy is a distal symmetrical polyneuropathy. Type 1 diabetic patients are diagnosed when beta-cell destruction is almost complete.

Streptozotocin is a toxin that has direct cytotoxic effects on β-cells when injected at high doses. STZ-treated mice lead to inflammatory autoimmune-mediated destruction of β-cells, developing overt diabetes, with marked hyperglycemia, hypoinsulinemia, polydipsia, and polyphagia.

Following the streptozotocin treatment the response threshold (g) is reduced considerably (Fig. 33). A dose-response effect of AS-19 was observed, with a clear antiallodynic effect in diabetic mice at 10 mg/kg (Fig 33).
Example 7: Carrageenan model of inflammatory pain

a) Effect of AS-19 on mechanical allodynia (measured by automatic von Frey) in acute inflammatory conditions (3 h post-carrageenan)

Three hours after carrageenan injection, prominent swelling was observed in the affected ipsilateral paw. Edema was accompanied by mechanical allodynia, as evidenced by reduced paw withdrawal threshold in the ipsilateral carrageenan-treated paw (right paw) respect to the control contralateral paw (left paw) in vehicle-treated mice (Fig 34). Systemic administration of AS-19 (10 mg/Kg, s.c.) 30 minutes before the von Frey test significantly reduced carrageenan-induced mechanical allodynia.

b) Effect of AS-19 on thermal hyperalgesia in chronic inflammatory conditions (4 days post-carrageenan)

Four days after carrageenan injection, swelling was still remarkable and thermal hyperalgesia was shown in the ipsilateral paw. A single injection with AS-19 (10 mg/Kg, s.c.) on day 4th significantly inhibited thermal hyperalgesia in the affected paw (Fig. 35). In fact, no thermal hyperalgesia was observed in mice receiving AS-19, as the withdrawal latency of the affected carrageenan-treated paw was not significantly different from that of the unaffected contralateral paw of the vehicle group.

Example 8: Formalin model of inflammatory pain.

Effect of AS-19 after formalin injection

AS-19 (5 mg/kg i.p.) exerted a significant decrease in paw licking time in the second phase of formalin test (Fig. 36).
CLAIMS

1. Use of a compound binding to the 5HT\textsubscript{7} receptor and acting as a full or partial agonist in the production of a medicament for the treatment of pain.

2. Use, according to Claim 1, in which the pain is neuropathic pain, especially hyperalgesia or allodynia.

3. Use, according to Claim 1, in which the pain is inflammatory pain.

4. Use, according to any of claims 2 or 3, characterized in that the stimulus evoking the pain is mechanical.

5. Use, according to any of claims 2 or 3, characterized in that the stimulus evoking the pain is thermal.

6. Use, according to claim 2, characterized in that the neuropathic pain, is selected from central pain, hyperpathia, peripheral neuropathic pain or peripheral neurogenic pain, causalgia, hyperesthesia, neuralgia, neuritis, or neuropathy.

7. Use, according to any of claims 1 to 6, characterized in that the compound binding to the 5-HT\textsubscript{7} receptor is binding with a higher affinity – expressed as a K\textsubscript{i}-value – to the 5-HT\textsubscript{7} receptor than to the 5-HT\textsubscript{1A} receptor; especially binding with an affinity higher by a factor of at least 10, preferably with an affinity higher by a factor of at least 30, more preferably with an affinity higher by a factor of at least 50, most preferably with an affinity higher by a factor of at least 100.

8. Use, according to any of claims 1 to 6, characterized in that the compound binding to the 5-HT\textsubscript{7} receptor is binding with a higher affinity – expressed as a K\textsubscript{i}-value – to the 5-HT\textsubscript{7} receptor than to the any other 5-HT receptor; especially binding with an affinity higher by a factor of at least 10, preferably with an affinity higher by a factor of at least 30, more preferably with an affinity higher by a factor of at least 50, most preferably with an affinity higher by a factor of at least 100.

9. Use, according to any of claims 1 to 6, characterized in that the compound binding to the 5-HT\textsubscript{7} receptor is binding to the 5-HT\textsubscript{7} receptor with a K\textsubscript{i}-value lower than 1000 nM, preferably lower than 200 nM, more preferably lower than 100 nM, even
more preferably lower than 50 nM, very preferably lower than 25 nM, most preferably lower than 10 nM.

10. Use, according to any of claims 1 to 9, characterized in that the compound binding to the 5-HT₇ receptor is binding to the 5-HT₇ receptor with a Ki-value lower than 1000 nM, preferably lower than 200 nM, more preferably lower than 100 nM, even more preferably lower than 50 nM, very preferably lower than 25 nM, most preferably lower than 10 nM

and

is binding with a higher affinity – expressed as a Ki-value – to the 5-HT₇ receptor than to the 5-HT₁₆ receptor; especially binding with an affinity higher by a factor of at least 10, preferably with an affinity higher by a factor of at least 30, more preferably with an affinity higher by a factor of at least 50, most preferably with an affinity higher by a factor of at least 100.

11. Use according to any of claims 1 to 9, characterized in that the compound binding to the 5-HT₇ receptor is binding to the 5-HT₇ receptor with a Ki-value lower than 1000 nM, preferably lower than 200 nM, more preferably lower than 100 nM, even more preferably lower than 50 nM, very preferably lower than 25 nM, most preferably lower than 10 nM

and

is binding with a higher affinity – expressed as a Ki-value – to the 5-HT₇ receptor than to any other 5-HT receptor; especially binding with an affinity higher by a factor of at least 10, preferably with an affinity higher by a factor of at least 30, more preferably with an affinity higher by a factor of at least 50, most preferably with an affinity higher by a factor of at least 100.

12. Use according to any of claims 1 to 11, characterized in that the compound binding to the 5-HT₇ receptor is acting as a full or partial agonist, especially a full agonist.

13. Use, according to any of claims 1 to 12, characterized in that the compound binding to the 5-HT₇ receptor is AS-19 or MSD5a, especially AS-19, optionally in the
form of its racemate, pure stereoisomers, especially enantiomers or diastereomers or in the form of mixtures of stereoisomers, especially enantiomers or diastereomers, in any suitable ratio; in the form shown or in form of the acid or base or in form of a salt, especially a physiologically acceptable salt, or in form of a solvate, especially a hydrate.
Figure 1:

HABITUATION
120 min

TREATMENTS
30 min

VON FREY STIMULATION
(1 g or 0.25 g)
15 min

CAPSAICIN (1 µg or 4 ng)
OR VEHICLE (1% DMSO)
Figure 2:

![Graph showing paw withdrawal latency comparison between Vehicle and Capsaicin 1 μg treatments](image)

- **Paw withdrawal latency (sec)**: 0 to 60
- **Vehicle**
- **Capsaicin 1 μg**

**Legend**:
- **VON FREY STIMULATION (1 g)**
- **HABITUATION**
- **120 min**
- **15 min**
- **CAPSAICIN (1 μg) OR VEHICLE (1% DMSO)**

Note: The graph indicates a significant difference in paw withdrawal latency between the Vehicle and Capsaicin 1 μg treatments, as indicated by the asterisks.
Figure 4:

Paw withdrawal latency (sec)

Vehicle 3 10 30
MSD-5a (mg/kg, sc)

120 min 30 min 15 min
HABITUATION
VON FREY STIMULATION (1 g)
CAPSAICIN (1 μg)

** ** *** ***
Figure 5:

Paw withdrawal latency (sec)

Vehicle  2.5  5  10  20
SB-258719 (mg/kg, sc)

***

HABITUATION
120 min

TREATMENTS
30 min

VON FREY STIMULATION
15 min
1 g

CAPSAICIN (1 μg)
Figure 6:

![Graph showing paw withdrawal latency (sec) vs dose (mg/kg, sc). The graph compares different treatments including Vehicle, SB-225719 (2.5), AS-19 (5), SB-225719 (2.5) + AS-19 (5), and SB-225719 (5) + AS-19 (5). Significant differences are indicated by ** and #.]

Dose (mg/kg, sc)

VON FREY STIMULATION (1 g)

HABITUATION

120 min 30 min 15 min

CAPSAICIN (1 µg)
Figure 7:

![Graph showing paw withdrawal latency with different doses and treatments.](image)

- **X-axis:** Dose (mg/kg, sc)
- **Y-axis:** Paw withdrawal latency (sec)

- **Vehicle**
- **SB-269970 (10)**
- **AS-19 (5)**
- **SB-269970 (10) + AS-19 (5)**

Significance markers indicate:
- **** for p < 0.01
- ### for p < 0.001

Timeline:
- **HABITUATION**
- **120 min**
- **30 min**
- **15 min**
- **CAPSAICIN (1 μg)**
- **TREATMENTS**
- **VON FREY STIMULATION (1 g)**
Figure 9:

![Graph showing the effects of WAY-100635 on paw withdrawal latency. The x-axis represents different doses of WAY-100635 (mg/kg, sc), and the y-axis represents the paw withdrawal latency (sec). The bars indicate the mean ± SEM, with * indicating statistical significance.]

- Vehicle: Baseline
- 0.1 mg/kg
- 0.3 mg/kg
- 0.5 mg/kg
- 1 mg/kg

![Timeline showing the study protocol: HABITUATION, TREATMENTS, VON FREY STIMULATION (1 g), and CAPSAICIN (1 μg). The timeline includes time points of 120 min, 30 min, and 15 min.]

- HABITUATION
- 120 min
- TREATMENTS
- 30 min
- CAPSAICIN (1 μg)
- 15 min
- VON FREY STIMULATION (1 g)
Figure 10:

Bar graph showing the effect of various doses on paw withdrawal latency. The x-axis represents the dose (mg/kg, sc), and the y-axis represents paw withdrawal latency (sec). The treatments include Vehicle, WAY-100635 (0.3), F-15640 (1), WAY-100635 (0.3) + F-15640 (1).

The graph indicates significant differences between the groups, with the highest latency observed in the WAY-100635 (0.3) + F-15640 (1) group.

Timeline below the graph:
- Habituation: 120 min
- Treatments: 30 min
- Von Frey stimulation: 15 min
- Capsaicin (1 μg)
Figure 11:

![Graph showing paw withdrawal latency (sec) vs. dose (mg/kg, sc) for different treatments: Vehicle, WAY-100635 (0.3), SB-239719 (5), AS-19 (5), AS-19 (10), SB-239719 (5) + AS-19 (10), WAY-100635 (0.3) + AS-19 (5), WAY-100635 (0.3) + AS-19 (10).](image)

Dose (mg/kg, sc)

HABITUATION | TREATMENTS | VON FREY STIMULATION (1 g)
---|---|---
120 min | 30 min | 15 min
CAPSAICIN (1 μg)
Figure 12:

![Graph showing the effect of different doses on paw withdrawal latency. The x-axis represents the dose (mg/kg, sc) and the y-axis represents the paw withdrawal latency (sec). The graph compares the effects of Vehicle, SB-258719 (10), SB-269970 (10), without capsaicin (vehicle=1% DMSO), and capsaicin (4 ng). The results show significant differences at different doses.]

- **Vehicle**
- **SB-258719 (10)**: Significantly higher paw withdrawal latency (*p < 0.001*)
- **SB-269970 (10)**: Significantly higher paw withdrawal latency (*p < 0.001*).

**VON FREY STIMULATION (0.25 g)**
- **HABITUATION**
- **TREATMENTS**
- **120 min**
- **30 min**
- **15 min**

**CAPSAICIN (4 ng) OR VEHICLE (1% DMSO)**
Figure 13:

![Graph showing paw withdrawal latency with different SB-258719 doses and time points.](image-url)
Figure 14:

Paw withdrawal latency (sec)

Vehicle
AS-19 (1)
AS-19 (9)
AS-19 (10)
SB-258719 (5)
SB-258719 (5) + AS-19 (1)
SB-258719 (5) + AS-19 (2)
SB-258719 (5) + AS-19 (5)
SB-269970 (5)
SB-269970 (5) + AS-19 (10)

VON FREY STIMULATION (0.25 g)

HABITUATION
TREATMENTS
120 min 30 min 15 min
CAPSAICIN (4 ng) OR VEHICLE (1% DMSO)
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<th>Day 5</th>
<th>Day 10</th>
<th>Days 11-13</th>
<th>Day 14</th>
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<td>Basal Pre-Injury</td>
<td>Surgery</td>
<td>Basal Post-Injury</td>
<td>Basal Pre-treatment (vehicle treatment)</td>
<td>Drug evaluation (drug treatment)</td>
<td>Basal Post-treatment (vehicle treatment)</td>
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<td>Habitation and measurements</td>
<td>Nerve injury or sham</td>
<td>Habitation and measurements</td>
<td>Habitation, treatment and measurements</td>
<td>Habitation, treatments (Latin square) and measurements</td>
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### Figure 16:

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<th>Day 5</th>
<th>Day 10</th>
<th>Day 11</th>
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<td>Surgery</td>
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<td>Nerve injury or sham</td>
<td>Habitation and measurements</td>
<td>Habitation, vehicle treatment and measurements</td>
<td>Habitation, drug treatment and measurements</td>
</tr>
</tbody>
</table>
**Figure 17:**

**A - NERVE LIGATURE**

*Withdrawal Threshold of Von Frey Filament Challenge (g)*

- **Dose (mg/kg, s.c.):**
  - Pre-Injury
  - Pre-Treatment
  - AS-19 (0.1)
  - AS-19 (0.3)
  - AS-19 (1)
  - AS-19 (5)
  - AS-19 (10)
  - Post-Treatment

---

**B - SHAM OPERATED**

*Withdrawal Threshold of Von Frey Filament Challenge (g)*

- **Dose (mg/kg, s.c.):**
  - Pre-Injury
  - Pre-Treatment
  - AS-19 (0.1)
  - AS-19 (0.3)
  - AS-19 (1)
  - AS-19 (5)
  - AS-19 (10)
  - Post-Treatment
Figure 18:

A - NERVE LIGATURE

![Graph showing paw withdrawal latency with different dose levels for Nerve Ligature treatment.]

B - SHAM OPERATED

![Graph showing paw withdrawal latency with different dose levels for Sham Operated treatment.]

Dosage (mg/kg, s.c.)
Figure 19:

**A - NERVE LIGATURE**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Withdrawal Threshold (g)</th>
<th>Dose (mg/kg, s.c.)</th>
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<td>PRE-TREATMENT</td>
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<tr>
<td>MSD-5a (0.9)</td>
<td>3.0 (*)</td>
<td></td>
</tr>
<tr>
<td>MSD-5a (1)</td>
<td>4.0 (***)</td>
<td></td>
</tr>
<tr>
<td>MSD-5a (9)</td>
<td>4.0 (***)</td>
<td></td>
</tr>
<tr>
<td>POST-TREATMENT</td>
<td>3.0 (*)</td>
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</table>

**B - SHAM OPERATED**

<table>
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<th>Dose (mg/kg, s.c.)</th>
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<td>PRE-TREATMENT</td>
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<td>MSD-5a (0.9)</td>
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<tr>
<td>MSD-5a (1)</td>
<td>4.5</td>
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<tr>
<td>MSD-5a (9)</td>
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<tr>
<td>POST-TREATMENT</td>
<td>4.5</td>
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</tbody>
</table>
**Figure 20:**

**A - NERVE LIGATURE**

![Bar chart showing paw withdrawal latency for different dose treatments in nerve ligature group.](chartA)

**B - SHAM OPERATED**

![Bar chart showing paw withdrawal latency for different dose treatments in sham operated group.](chartB)
Figure 21:

A - NERVE LIGATURE

B - SHAM OPERATED
Figure 22:

A - NERVE LIGATURE

B - SHAM OPERATED
Figure 23:

- Top graph:
  - Pre-injury
  - Pre-treatment
  - SB-258719 (5)
  - AS-19 (5)
  - AS-19 (5) + SB-258719 (5)

- Bottom graph:
  - Pre-injury
  - Pre-treatment
  - SB-258719 (5)
  - MSD-5a (10)
  - MSD-5a (10) + SB-258719 (5)
Figure 25:

CCI-SN, Paw withdrawal threshold

Randall-Selitto test

CCI-SN, Vocalization threshold

- ○ saline + AS 19 2.5 mg/kg s.c. (n = 5-8)
- • saline + AS 19 5 mg/kg s.c. (n = 5-8)
- ■ saline + AS 19 10 mg/kg s.c. (n = 5-13)
Figure 26:

CCI-SN, Paw withdrawal threshold

Randall-Selitto test

CCI-SN, Vocalization threshold

- saline + 5a-MSD 5 mg/kg s.c. (n=6)
- saline + 5a-MSD 10 mg/kg s.c. (n=9)
- saline + 5a-MSD 15 mg/kg s.c. (n=6)
Figure 27:

CCI-SN, Paw withdrawal threshold

CCI-SN, Vocalization threshold

- saline + AS 19 10 mg/kg s.c. (n=8)
- SB 269970 3 mg/kg i.p. + saline (n=6)
- SB 269970 3 mg/kg i.p. + AS 19 10 mg/kg s.c. (n=5)
Figure 28:

CCI-SN, Paw withdrawal threshold

CCI-SN, Vocalization threshold

- saline + 5a-MSD 10 mg/kg s.c. (n=9)
- SB 258719 + saline (n=2)
- SB 258719 3 mg/kg + 5a-MSD 10 mg/kg s.c. (n=6)
Figure 30:

CCI-IoN

Von Frey filaments

Pressure (g)

Time (min)

- saline + 5a-MSD 5 mg/kg s.c. (n=3)
- saline + 5a-MSD 10 mg/kg s.c. (n=6)
- saline + 5a-MSD 15 mg/kg s.c. (n=6)
- sham saline + 5a-MSD 10 mg/kg s.c. (n=4)
Figure 31:

CCI-Ion

Von Frey filaments

![Graph showing pressure over time with different treatment groups and statistical significance markers.]

- saline + AS 19 10 mg/kg s.c. (n=8)
- SB 269970 3 mg/kg i.p. + saline (n=5)
- SB 269970 3 mg/kg i.p. + AS 19 10 mg/kg s.c. (n=6)
Figure 32:

CCl-IoN

Von Frey filaments

Pressure (g)

Time (min)

-●- saline + 5a-MSD 10 mg/kg s.c. (n=6)
-●- SB 269970 3 mg/kg i.p. + 5a-MSD 10 mg/kg s.c. (n=4)
Figure 33:

![Graph showing withdrawal threshold for Von Frey Filament Challenge over different days and treatment groups.]

- **Vehicle**
- **AS-19 (1)**
- **AS-19 (5)**
- **AS-19 (10)**
Figure 34:

- **Paw withdrawal threshold (g)**
- **Vehicle**
- **AS-19 (10)**

- Contralateral paw
- Ipsilateral paw

***

***

##

##

*
Figure 36:

- **Vehicle**
- **AS-19 (5)**

The graph shows the paw licking time (sec) for different treatments. The bar for AS-19 (5) is significantly lower than the bar for Vehicle, indicated by the *** symbol.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/00 A61K31/415 A61K31/4418 A61P29/00

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)
A61K A61P

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, CHEM ABS Data, WPI Data, EMBASE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>page 2, lines 65-67; claims 1-13; table 1</td>
<td>3,13</td>
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<td>X</td>
<td>ARULMOZHI ET AL: &quot;Migraine: Current concepts and emerging therapies&quot; VASCULAR PHARMACOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 43, no. 3, September 2005 (2005-09), pages 176-187, XPO05029721 ISSN: 1537-1891</td>
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* Further documents are listed in the continuation of Box C.
X See patent family annex.

* 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
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Date of the actual completion of the international search
23 October 2007

Date of mailing of the international search report
12/11/2007

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