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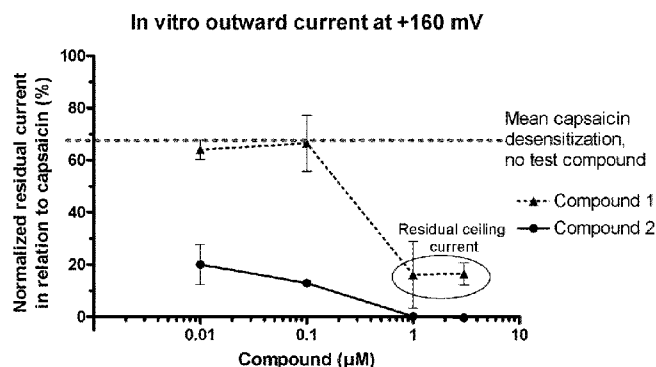


Figure 1

(57) Abstract: The invention relates to an in vitro screening assay based on electrophysiological recording techniques for identifying thermoneutral TRPV1 modulators comprising a) providing a cell expressing TRPV1, optionally in contact with a suitable concentration of a TRPV1 agonist under suitable conditions, b) providing said cell in contact with a suitable concentration of a TRPV1 modulator and optionally in contact with a TRPV1 agonist, if present in step a), under suitable conditions, c) applying a voltage above +100 mV to the cells of a) and of b), d) measuring the respective current of the cells' membranes of a) and b), and e) determining the change of the value of b) compared to the value of a), whereby in the presence of a TRPV1 agonist a residual current of 5 to 50% of b) relative to a) is indicative of a TRPV1 modulator being thermoneutral, and whereby in the absence of a TRPV1 agonist the indication of a TRPV1 modulator being thermoneutral is based on the measurement of the deviation of the current of a) relative to b), whereby a deviation in the range of 50% is indicative of a TRPV1 modulator being thermoneutral, as well as to the use of said screening assay for characterizing the thermogenetic properties of a TRPV1 modulator.

In Vitro Screening Assay for Thermoneutral TRPV1 Modulators

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The present invention relates to an in vitro screening assay for thermoneutral TRPV1 modulators. More specifically, the present invention relates to an in vitro screening method useful in the identification of TRPV1 modulators that have no effect on the thermoregulation of an organism. Said screening assay allows for the rapid screening of a large number of compounds by providing a simple conduct of the method that can be easily automated.

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Transient Receptor Potential channel vanilloid type 1 (TRPV1) is an excitatory, non-selective cation channel of the TRP superfamily of ion channels. TRPV1 is expressed in various mammalian tissues, including the peripheral and central nervous system, where it can be directly gated by endogenous stimuli (various lipids, such as anandamide), low pH and membrane depolarization, or exogenous stimuli like heat ($>43^{\circ}\text{C}$) and vanilloid compounds like capsaicin (the "hot" ingredient in chilli peppers). Due to its sensitivity for various modalities, physical and chemical stimuli, TRPV1 has been designated a polymodal receptor.

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TRPV1 activation leads to monovalent (sodium) and divalent (calcium) cation influx that induces membrane depolarization, triggers calcium-dependent intracellular transduction cascades, induces neuronal firing of action potentials and leads to neuronal release of inflammatory mediators like CGRP and Substance P. Sensory dorsal root and trigeminal ganglion neurons that express TRPV1 can be characterised as nociceptors, mediating a hot and pungent pain sensation when TRPV1 is stimulated. In addition to its acute nociceptive capability, inflammatory and neuropathic conditions sensitise TRPV1, leading to hypersensitivity.

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Studies with knock-out mice have underlined a role for TRPV1 in heat and mechanical hyperalgesia and allodynia. The inherent role in pain perception has encouraged efforts to develop TRPV1 antagonists in order to block pathological pain. In recent years potent, small molecules have been identified and tested preclinically and clinically for their analgesic efficacy. However, these experiments also revealed that such compounds have tar-

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get-related side-effects, an increase of body temperature (i.e., hyperthermia) due to TRPV1 block (Bannon et al., Society for Neuroscience Annual Meeting 2004; Gavva et al., Neurosci. 2007b, pp. 3366-74; Swanson et al., J. Med. Chem. 2005, pp. 1857-72). A variety of selective TRPV1 antagonists, representing various chemotypes, caused hyperthermia in rats within 30-90 min after their administration (Gavva et al., Neurosci. 2007b, pp. 3366-74). The search for the mechanism of action underlying such body temperature effects suggests that, under non-pathological conditions, TRPV1 has a temperature-dependent constitutive activity in peripheral sensory and/or central hypothalamic neurons and that antagonism of TRPV1 simulates a decrease of body temperature in neurons that are involved in core body temperature regulation (Gavva et al., loc. cit.). As a consequence, complex physiological mechanisms lead to an increase of body temperature. Repeated dosing of TRPV1 antagonists revealed the development of tolerance for hyperthermia but not for analgesia (Honore et al., Pain 2009, pp. 27-35; Gavva et al., J Pharmacol Exp Ther. 2007a, pp. 128-37). This effect might be exploited for the development of a thermoactive TRPV1 blocker but still holds the risk of hyperthermia in single individuals. Another conceivable side-effect of TRPV1 antagonism could be a disturbed perception of temperature on the skin and mucous membranes of the airways and oral/nasal cavity.

Although effects on body temperature regulation have been clearly demonstrated in vivo, its molecular determinants on the level of the TRPV1 receptor are largely unclear. During the last decade, biophysical characterisation has identified sensitivity of channel gating for the cellular membrane potential as an outstanding feature of TRPV1. Depolarisation [i.e., change of voltage from the neuronal resting membrane potential (~ -80 mV) to positive potentials] results in channel activation.

Electrophysiological recording techniques, the gold standard for ion channel investigation that allows precise analysis of voltage-dependent channel gating, were utilised extensively to characterise channel activation and grounded the present hypothesis that various endogenous and exogenous stimulants, like capsaicin, pH and heat, act by a single mechanism: the shift of the voltage dependent activation to more negative potentials (Voets et al., Nature 2004, pp. 748-54; Nilius et al., J Physiol 2005, pp. 35-44). In consequence, at chemical or temperature stimulation, negative membrane potentials (near the resting membrane potential) become sufficient to activate TRPV1 channels, leading to channel opening and cellular excitation. Electrophysiological measurements furthermore revealed that voltage-dependent activation gradually depends on the dose of chemical stimulation

and the intensity of heat stimulation. For the latter, the current hypothesis claims that, at body temperature, TRPV1 channels may have an activity that is dynamically modulated by small changes in temperature and membrane potential (Gavva et al., loc. cit.). Potent antagonists typically shift the voltage-dependent channel activation to more positive potentials and result in a full channel block – also at very positive membrane potentials that practically will not be reached in a cell. Such potent antagonists suppress the channel activity at body temperature, an effect that may, in its global physiological context, induce hyperthermia.

So far it has not been possible to identify a compound's thermogenetic profile by means of an in vitro assay that predicts thermoneutrality in vivo and that is applicable to TRPV1 modulators to be administered to human beings. So far the actual thermogenetic properties of TRPV1 modulators may effectively only be tested in animal experiments. Said experiments, however, are disadvantageous insofar as they do not account for species differences concerning the thermoregulation. Given the fact that the mode of thermoregulation varies to a large extent from species to species, animal experiments only have a limited significance, i.e., they are only significant as far as the tested species is concerned and may not reliably be transferred to the situation in any other or a human organism. To date, no studies on TRPV1 antagonists which are thermoneutral in humans have been published.

There is, therefore, a need for a screening test that allows the characterisation of TRPV1 modulators in terms of their thermogenetic properties and that allows the identification of thermoneutral TRPV1 modulators, preferably TRPV1 antagonists. Said assay should allow a reliable determination of the thermogenetic properties of TRPV1 modulators intended to be given to various mammalian and other species including human beings. Moreover, the assay should allow for many compounds to be screened quickly, should be automatable, easy to use, sensitive and selective. Said assay should also provide a high signal-to-background-noise ratio allowing the obtention of reliable results and leading to a clear identification of thermoneutral TRPV1 modulators. The screening test should be suitable for high throughput screening.

According to the present invention, it has surprisingly been found that TRPV1 modulators that do not have the effect of increasing the body temperature, i.e., that do not cause hyperthermia, can be found by evaluating the residual activation properties (residual outward

current, residual ceiling current, ceiling residual current) upon voltage-dependent activation at very positive unphysiological membrane depolarisation above +100 mV, and that even at room temperature strong depolarisation is sufficient to identify compounds with thermoneutral characteristics by a residual outward current.

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Accordingly, the present invention relates to an in vitro screening assay based on electrophysiological recording techniques for identifying thermoneutral TRPV1 modulators comprising a) providing a cell expressing TRPV1, optionally in contact with a suitable concentration of a TRPV1 agonist under suitable conditions, b) providing said cell in contact with a suitable concentration of a TRPV1 modulator and optionally in contact with a TRPV1 agonist, if present in step a), under suitable conditions, c) applying a voltage above +100 mV to the cells of a) and of b), d) measuring the respective current of the cells' membranes of a) and b), and e) determining the change of the value of b) compared to the value of a), whereby in the presence of a TRPV1 agonist a residual current of 5 to 50% of b) relative to a) is indicative of a TRPV1 modulator being thermoneutral, and whereby in the absence of a TRPV1 agonist the indication of a TRPV1 modulator being thermoneutral is based on the measurement of the deviation of the current of a) relative to b), whereby a deviation in the range of 50% is indicative of a TRPV1 modulator being thermoneutral.

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The present invention allows identification of thermoneutral TRPV1 modulators and in particular thermoneutral TRPV1 analgesics in vitro even at room temperature by exploiting inherent biophysical properties of the TRPV1 ion channel. A feature of TRPV1, fundamental for the present invention, is its sensitivity for cellular membrane depolarisation: Change of voltage from a cellular (negative) membrane potential to positive potentials results in channel activation. Using the electrophysiological patch-clamp techniques, this depolarisation-induced activation manifests in an outward (by definition positive) current. Most TRPV1-inhibiting compounds that have been developed in mice block these outward currents completely. In preceding detailed studies by the inventors of the invention at hand, compounds that were characterised as thermoneutral in mice were tested for voltage-dependent activation of mouse TRPV1 in vitro. This analysis revealed residual activation properties (residual outward current) at very positive, unphysiological membrane depolarisation >100 mV, even at very high compound concentrations. In contrast to these thermoneutral compounds, hyperthermia-inducing compounds completely abolish outward currents. Additional experiments showed that thermoneutral compounds largely block chemical, capsaicin-induced activation but have a minor effect on the interaction with

temperature-dependent gating allowing a dynamic “thermo-modulatory gating window”. The invention discloses that even at room temperature strong depolarisation is sufficient to identify compounds with “thermoneutral” characteristics by a “residual outward current”.

5 According to the present invention, TRPV1 modulators are identified in vitro that do not induce hyperthermia in vivo (hereafter referred to as “thermoneutral compounds”) by applying automated electrophysiological recording techniques (patch-clamp), preferably under room temperature conditions. The assay of the present invention allows an in vitro screening for “thermoneutral” TRPV1 modulators, using a common heterologous expres-
10 sion system (for example, HEK293 cells) preferably in combination with an automated patch-clamp platform (for example, QPatch 16, Sophion, Copenhagen, Denmark). As defined above, the present invention is primarily based on the inventors’ discovery that thermoneutral compounds reveal characteristic inhibitory effects on TRPV1 in vitro. These inhibitory properties are characterised (at room temperature) by a partial antagonism at
15 very positive, for ion channels unphysiological, membrane potentials. In contrast, hyperthermia-inducing compounds completely block ion channel activity at all membrane potentials that can be measured by means of patch-clamp recordings. The partial antagonism of the thermotolerant compounds may be based on their specificity for modality, e.g., chemical activation by capsaicin is blocked but temperature modulation of TRPV1 is
20 less/not affected. Selected compounds were tested by manual patch-clamp for their impact on temperature-dependent modulation of TRPV1. These experiments revealed that TRPV1 inhibition by thermoneutral compounds is specific for modality, e.g., chemical activation by capsaicin is blocked but temperature modulation of TRPV1 is less/not affected. In automated patch-clamp experiments, the low (room) temperature, e.g., of 23°C proved
25 to be sufficient to activate heterologously expressed TRPV1 at very positive membrane potentials.

It is noted that whereas many TRPV1 modalities have been analysed by the patch-clamp technique, high throughput analysis of temperature modulation has rather seldom been
30 conducted with this technique probably due to the fact that usual automated patch-clamp platforms do not allow changes in assay temperatures and usually studies are conducted at room temperature. The present invention, therefore, for the first time describes an electrophysiological assay which is conducted at room temperature and which exploits the voltage-dependent activation characteristics of TRPV1 so as to allow the identification of

compounds that have no or only a minor effect on the temperature-dependent modulation while retaining the full capacity to be potent inhibitors for chemical stimulation.

5 According to the in vitro screening assay of the present invention, in a first step a cell expressing TRPV1 is provided and is optionally contacted with a suitable concentration of a TRPV1 agonist under suitable conditions.

10 Cells for use in the assay of the invention contain a functional TRPV1 or a functional fragment thereof. The person skilled in the art may use cells in which TRPV1 is endogenous or may introduce TRPV1 into a corresponding cell. TRPV1 may be expressed in any type of cell, preferentially in a cell line for heterologous expression that preferentially allows a high expression rate. The TRPV1 receptor may be stably integrated or may be transiently expressed in any suitable cell. Corresponding cells are either available in the art or can easily be prepared by a person skilled in the art using standard techniques of
15 molecular cloning and expression. Examples of cells that easily can be prepared for transient or stable expression of TRPV1 are HEK293 and CHO cells. An example for endogenously TRPV1 expressing cells are dorsal root ganglion (DRG) neurons.

20 The TRPV1 receptor to be used according to the present invention may be of any species origin and preferably of any mammalian species origin. The receptor is preferably of human origin.

25 The cells for use in the assay of the invention are usually present in an appropriately detached (in suspension) form but may also be present in any form that is suitable for an electrophysiological measurement.

30 As a TRPV1 agonist, any compound having an agonistic effect (i.e., causing the current to increase) on TRPV1 may be used. Examples of TRPV1 agonists are capsaicin, resiniferatoxin, olvanil, arvanil, SDZ-249665, SDZ-249482, nuvanil and capsavanil, NADA, pH, heat. Preferably, the TRPV1 agonist is capsaicin.

A suitable concentration of a TRPV1 agonist is a concentration that leads to a full or nearly full or a partial agonistic effect on the respective TRPV1 receptor.

Suitable conditions for contacting a cell expressing TRPV1 with a TRPV1 agonist are conditions under which the TRPV1 and the agonist have the usual physiological effect, i.e., resembling the physiologically acceptable conditions for receptor-agonist interaction in terms of buffer composition, pH value etc. depending on the kind of cell as used.

The step of contacting a cell expressing TRPV1 with a suitable concentration of a TRPV1 agonist under suitable conditions in the first step of the assay may be omitted but is preferably carried out to enhance the sensitivity of the assay, since said step increases the current to be measured on the cells.

The cell expressing TRPV1 is then contacted with a suitable concentration of a TRPV1 modulator and a TRPV1 agonist, if present in step a) under suitable conditions.

A TRPV1 modulator basically is any compound that has an effect on the activity of TRPV1. This effect can be agonistic, antagonistic, partially agonistic or partially antagonistic or a combination of said effects. TRPV1 modulators are known in the art and may, according to the present invention, be tested on their respective thermogenetic properties. The assay of the invention, however, is also applicable to new TRPV1 modulators, the TRPV1-modulating property of which as such is tested in a known TRPV1 gating assay and the thermogenetic property of which is tested in the assay of the present invention.

Examples of TRPV1 modulators are natural ligands like

(*E*)-*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-enamide (Capsaicin), Resiniferatoxin and

N-arachidonyl dopamine (NADA), certain amide analogues like 1-(3-(trifluoromethyl)pyridin-2-yl)-*N*-(4-(trifluoromethylsulfonyl)phenyl)-1,2,3,6-tetrahydropyridine-4-carboxamide (A-784168) and

5-(4-chlorophenylcarbamoyl)-2-(isoquinolin-5-yl)pyridine 1-oxide (V-405), certain cinnamide analogues like (*E*)-3-(4-chlorophenyl)-*N*-(3-methoxyphenyl)acrylamide (SB-366791),

(*E*)-3-(4-*tert*-butylphenyl)-*N*-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)acrylamide propenamide (AMG-9810) and

(*R,E*)-*N*-(2-hydroxy-2,3-dihydro-1*H*-inden-4-yl)-3-(2-(piperidin-1-yl)-4-

(trifluoromethyl)phenyl)acrylamide (AMG-8562), certain urea analogues like *N*-(4-*tert*-butylphenyl)-4-(3-chloropyridin-2-yl)piperazine-1-carboxamide (BCTC), 4-(3-

(trifluoromethyl)pyridin-2-yl)-*N*-(5-(trifluoromethyl)pyridin-2-yl)piperazine-1-carboxamide

(JNJ-17203212), 1-(isoquinolin-5-yl)-3-(4-(trifluoromethyl)benzyl)urea (A-425619), (*S*)-1-(2-bromophenyl)-3-(1-(5-(trifluoromethyl)pyridin-2-yl)pyrrolidin-3-yl)urea (SB-705498) and (*R*)-1-(5-tert-butyl-2,3-dihydro-1H-inden-1-yl)-3-(1H-indazol-4-yl)urea (ABT-102), certain pyrimidine or quinazoline analogues like

N-(4-(6-(4-(trifluoromethyl)phenyl)pyrimidin-4-yloxy)benzo[d]thiazol-2-yl)acetamide (AMG-517) and

N-(4-(trifluoromethyl)phenyl)-7-(3-(trifluoromethyl)pyridin-2-yl)quinazolin-4-amine (NRGN-16).

Preferably, a TRPV1 modulator to be tested is a TRPV1 antagonist.

The respective compound is tested in a concentration suitable to have an effect on the activity of TRPV1. A suitable concentration of a TRPV1 modulator is any concentration that is physiologically acceptable with respect to the TRPV1 receptor and the cell as used and leads to a meaningful result in terms of the electrophysiological properties of that cell.

The most favourable results in the assay of the present invention are obtained when the compound to be tested is applied in high concentrations, i.e., concentrations leading to a ceiling effect in a dose-response relation in the claimed assay. As a rough guideline to a person skilled in the art, the concentration to be used in the present assay is about 100 to 1,000 times the concentration that reveals a half maximal effect in classical TRPV1 assays. A suitable concentration range comprises the range of 1 nmol to 1 mmol. A person skilled in the art will easily be in the position to determine the optimal concentration to be used in the assay of the present invention by applying routine experiments along the above rationale.

If in the first step a TRPV1 agonist is present, then the same or a different TRPV1 agonist is present in the second step of the assay. Preferably, the assay is carried out in the presence of a TRPV1 agonist. Preferably, the same TRPV1 agonist is given in both steps of the assay. Said agonist is given in a suitable concentration as defined above.

Preferably, the TRPV1 modulator and the TRPV1 agonist are applied simultaneously to the cell. Suitable conditions for contacting a TRPV1-expressing cell with a TRPV1 modulator are conditions allowing for the usual physiological reactions between said compounds in terms of buffer composition, pH value etc. depending on the kind of cell as used.

In a further step an unphysiologically high voltage, i.e., a voltage in a range that is beyond the voltage range that usually occurs in the respective species organism, is applied to said cells. Preferably, the voltage is above +100 mV, preferably above +140 mV, and may reach up to any value that is physiologically acceptable to the respective cell to be tested.

5 A preferred range of voltage values is between +100 mV and +200 mV. Preferably, the voltage is + 160 mV.

The voltage may be applied as a voltage jump, a permanent holding potential or a voltage ramp. Preferably, the voltage is applied as a voltage jump. The voltage is applied for a

10 time suitable to allow measurement of the respective current of the cells. Preferably, the voltage is applied for 1 ms to 1 s, more preferably 50 ms to 800 ms, most preferably 400 ms to 600 ms.

The voltage is applied by any technique allowing to impose a voltage to cells/cell membrane/ artificial lipid bilayers in whole-cell/ excised patch/ on-cell mode and allowing measurement of ion flow through membranes/ the ion channel. Suitable techniques are, e.g., the patch-clamp technique with voltage-clamp. Current-clamp may also be applied with the patch-clamp technique to depolarize cells/cell membrane/ artificial lipid bilayers and to determine ion channel activity under depolarized membrane potentials. A person

15 skilled in the art will be aware of the fact that when using the current-clamp technique the present assay is to be modified in that a current is applied to the cells and the respective voltage is measured on the cells. Preferably, the patch-clamp technique with voltage-clamp is used.

As a next step the respective currents on the cell membranes are measured and the values are compared, i.e., changes are determined. A residual current of the cell contacted with the compound to be tested in the presence of TRPV1 agonist of <5% is indicative of a hyperthermic effect, whereas a residual current of 5 to 50%, preferably 5 to 40%, more preferably 10 to 30%, in relation to the cell without test compound but under the effect of a

25 TRPV1 agonist is characteristic of thermoneutral modulators (test compounds). When the assay is carried out in the absence of a TRPV1 agonist, a complete blockage of the current is indicative of a hyperthermic compound, so that said embodiment of the invention allows an easy recognition of hyperthermic compounds.

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In said embodiment of the invention the referential value is the current value of the depolarised TRPV1-expressing cell without any test compound. Compounds that, in absence of agonist stimulation, have a minor effect on voltage activated channel activity but reveal antagonistic properties in standard alternative assays, may also be thermoneutral in vivo.

Any deviation of the current of the cells in the presence of the compound to be tested from the current of the TRPV1 expressing cell alone is indicative of the test compound to be thermoneutral. Preferably, a deviation of 50%, more preferably of 20% in the respective currents denotes thermoneutrality of a compound.

It is the major advantage of the screening assay according to the present invention that it can be carried out at room temperature. Therefore, said assay is highly suitable for automated high throughput screenings but may, of course, also be used on a scale of manual patch-clamp.

The temperature to be applied may be applied immediately or gradually (temperature ramp). It is possible to carry out the screening assay according to the present invention at any temperature that is physiologically acceptable for the cells expressing TRPV1. Basically, any temperature above the freezing temperature of said cells until the highest possible temperature that does not destroy the cells is possible. A preferred temperature range is between 5°C and 60°C. Preferably, the assay is carried out at a temperature between 20°C and 25°C. A further suitable temperature is 37°C.

A further major advantage of the present invention is the fact that the assay can specifically be carried out on the TRPV1 receptor of the species to which the TRPV1 modulator is to be given later. This means that the results obtained in the assay according to the present invention are transferable to the physiological situation in the respective organism. If, for example, thermogenetic properties of a TRPV1 modulator are tested on a cell expressing a human TRPV1 receptor, the results obtained are valid for the situation in the human organism. This means that time-consuming and laborious animal experiments of the prior art may conveniently be replaced by the screening assay according to the present invention with the additional advantage that a more direct, reliable and pharmacologically useful result is obtained concerning the respective TRPV1 modulator.

Further embodiments, features and advantages of the present invention are described in detail below with reference to the accompanying figures.

The accompanying figures illustrate embodiments of the present invention and together with the description further serve to explain the principle of the present invention.

5 Figure 1 shows the dose (concentration) response relation of two different compounds measured at +160 mV on murine TRPV1.

Figure 2 shows in vivo experiments in mice, characterising the effect of different doses of compounds on the body temperature 10 minutes after intravenous (i.v.) treatment. Base-
10 line shows the body temperature after vehicle treatment. Data are shown as °C (mean $\pm$ SEM).

In the following specific embodiments of the invention are described.

15 The following protocol has proved suitable to carry out the assay of the invention and leads to particularly favourable results. Variations of said protocol within the scope of the present invention are, of course, easily possible and will be apparent to a person skilled in the art.

20 In one embodiment of the invention the QPatch 16 device was applied to characterise modulation of voltage-dependent activation of murine TRPV1 by in mice thermoneutral compounds in comparison to hyperthermia-inducing compounds. Cells were kept under conventional cell culture conditions and were applied to the QPatch 16 in suspension. All experiments with the QPatch 16 were conducted at room temperature. Standard extracel-
25 lular (EC) and intracellular (IC) solutions were used [EC (in mM): 150 NaCl, 2 CaCl₂, 10 HEPES, pH 7.3, ~330 mOsm; IC (in mM): 135 NaF, 15 NaCl, 5 EGTA, 10 HEPES, pH 7.3, ~315 mOsm]. The experimental sequence was designed by a (1) "whole cell protocol" in order to define the conditions for automated whole-cell access according to common electrophysiological knowledge and according to suggestions from Sophion for the usage of
30 the QPatch 16, (2) "voltage protocol" in order to define the depolarizing voltage-step, and (3) "application protocol" for defining the sequential application of an agonist, alone and in combination with the test compound. Conveniently, said protocols may be programmed in a suitable software program and may be carried out automatically. Said steps, however, may also be carried out with a manual or semi-automatic patch-clamp setup or semi-
35 automatically. Preferably, the whole protocol is carried out automatically.

(1) The "whole cell" protocol defines parameters for the automated treatment of cells and the identification of valid "whole-cell" measurements. These settings and parameters are common electrophysiological knowledge and are not particularly specific for the assay of the invention.

(2) The "voltage protocol" defines the conditions of the determination of the membrane potential. For detection of the resulting residual ceiling current strong depolarization has to be applied, preferentially a voltage jump from the holding potential (preferentially -80 mV) to preferred > 100 mV, more preferred > 160 mV for 600 ms. The more positive the voltage, the better the assay sensitivity. The strength of depolarisation is solely limited by cell patch stability. The outward current amplitudes (preferentially steady state currents) were the measure. These amplitudes were analyzed in the presence of potential modulators. Setting a large, for ion channels unphysiological membrane depolarization is crucial for the present invention.

(3) The "application protocol" defines the sequential application of modulators (delivered in the extracellular solution) and the onset of the voltage protocol. All liquids are usually applied shortly (within minutes) before running the voltage protocol. TRPV1 receptor expression is monitored by a significant increase of the voltage-induced outward current by application of an agonist like capsaicin, resiniferatoxin, olvanil, arvanil, SDZ-249665, SDZ-249482, nuvanil and capsavanil, NADA, pH, heat.

Then the test compound is co-applied with the agonist in order to evaluate the modulation effect on chemically stimulated TRPV1. Finally, a full receptor block may be done using a potent blocker like 1 μ M N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC). Complete block of TRPV1 mediated outward currents identified non-TRPV1-related currents (e.g., from leak or not specified endogenous ion channels) and is, thus, a convenient tool to enhance the accuracy of the measurement, especially in case of the presence of other non-TRPV1-related ion channels on the cells. For data analysis all currents are normalized for 100 nM capsaicin (current under capsaicin treatment = 100%) and 1 μ M BCTC (current under BCTC = 0%).

Outward current in presence of capsaicin and the test compound is the measure and reveals the "residual outward current" when thermoneutral but not hyperthermic compounds

are applied. Test compounds shall always be used at high concentration that allows a maximal inhibitory effect, meaning a concentration within the “ceiling” range of the dose response relation. Since TRPV1 inhibition usually is voltage-dependent (release of block by membrane depolarization), at +160 mV very high compound concentrations may be necessary and some compounds do not display “ceiling” up to the highest concentration possible. Therefore, dose-response experiments are usually performed, herein with 0.1, 0.3, 1, and 3 μ M and only compounds that reveal “ceiling” of the dose response relation, herein at 1 and 3 μ M, will be evaluated using this method. Compounds, that reveal a “ceiling residual current” on murine TRPV1 of less than 40% in comparison to the capsaicin stimulation also show minor or no effects on body temperature in mice.

Due to receptor desensitization in vitro following capsaicin application, control experiments are usually performed with capsaicin alone that reveals a mean outward current of ~70%. A test compound that like capsaicin alone reveals a “ceiling current” of ~70% therefore has not a blocking effect but rather may have agonistic potency. No “ceiling current” predicts hyperthermic effects in vivo while a “ceiling current” of 1 to 50%, preferentially 5 to 40% , more preferentially 10 – 30% is typical for thermoneutral modulators.

Since so far no TRPV1 modulators that have no effect on the thermoregulation of a human organism have been described in the art, the present invention provides the first high throughput screening method useful for the identification of thermoneutral TRPV1 modulators. The assay according to the present invention is highly selective for thermoneutral TRPV1 modulators and allows a practitioner to distinguish between TRPV1 modulators that have an effect on the thermogenesis of an organism and those that do not have such an effect.

The compounds identified by the method of the present invention are useful as analgesics, not having the unwanted side-effect of causing hyperthermia. These TRPV1 modulators may be used for the treatment and/or prophylaxis of one or several diseases of the group of pain, preferentially of pain of the group of acute pain, chronic pain, neuropathic pain, visceral pain, arthralgia, hyperalgesia, allodynia, kausalgia and migraine.

The assay of the present invention can be used to identify new thermotolerant TRPV1 modulators but may also be used to characterise known TRPV1 modulators in terms of their thermogenetic properties.

Examples

Example 1

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Several TRPV1 antagonists that induce hyperthermia in mice were developed by the applicant (Compound 3, Compound 4) and other companies (AMGEN: AMG-981, AMG6880) and were tested according to the above-described embodiment for a “residual ceiling current”, i.e., for their ability to induce hyperthermia. All these hyperthermic compounds showed no/a small “residual ceiling current” (<3%) when activated by depolarization to +160 mV and given in presence of 100 nM capsaicin. Currents were normalized to the current from a preceding depolarization under 100 nM capsaicin alone and subtraction of leak/ not TRPV1 related currents identified by application of BCTC. As example, Compound 2 is shown in Figure 1 and 2. In contrast, compounds with a minor effect on body temperature in mice) showed a “residual ceiling current” of 10 to 30%. Compound 1 is shown as an example in Figures 1 and 2.

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Male NMRI mice were randomly assigned to the treatment groups with a group size of n=5. Body temperature was determined by means of a rectal temperature probe before and after intravenous (i.v.) administration of compound or vehicle (10% DMSO, 5% Cremophor EL in 5% glucose solution).

C L A I M S

5 1. In vitro screening assay based on electrophysiological recording techniques for identifying thermoneutral TRPV1 modulators comprising

- 10 a) providing a cell expressing TRPV1, optionally in contact with a suitable concentration of a TRPV1 agonist under suitable conditions,
- b) providing said cell in contact with a suitable concentration of a TRPV1 modulator and optionally in contact with a TRPV1 agonist, if present in step a), under suitable conditions,
- c) applying a voltage above +100 mV to the cells of a) and of b),
- 15 d) measuring the respective current of the cells' membranes of a) and b), and
- e) determining the change of the value of b) compared to the value of a),

whereby in the presence of a TRPV1 agonist a residual current of 5 to 50% of b) relative to a) is indicative of a TRPV1 modulator being thermoneutral, and whereby in the absence
20 of a TRPV1 agonist the indication of a TRPV1 modulator being thermoneutral is based on the measurement of the deviation of the current of a) relative to b), whereby a deviation in the range of 50% is indicative of a TRPV1 modulator being thermoneutral.

25 2. The assay of claim 1, wherein in steps a) and b) a TRPV1 agonist is present and the agonists are the same.

3. The assay of claim 2, wherein the agonist is capsaicin.

30 4. The assay of one of claims 1 to 3, wherein a voltage of +100 to +200 mV is applied to the cells.

5. The assay of claim 4, wherein a voltage of +160 mV is applied to the cells.

35 6. The assay of one of claims 1 to 5, wherein the voltage is applied as a voltage jump, permanent holding potential or a voltage ramp.

7. The assay of one of claims 1 to 6, wherein the voltage is applied for a time between 1 ms to 1 s.

5 8. The assay of one of claims 1 to 7, wherein the assay is carried out at a temperature between 5°C and 60°C.

9. The assay of claim 8, wherein the assay is carried out at room temperature.

10 10. The assay of one of claims 1 to 9, wherein the cell expressing a TRPV1 is a homologous or heterologous cell into which the TRPV1 receptor is stably integrated or in which the TRPV1 receptor is transiently expressed.

11. The assay of one of claims 1 to 10, wherein the TRPV1 receptor is a human TRPV1
15 receptor.

12. The assay of one of claims 1 to 11, wherein the TRPV1 modulator is a TRPV1 antagonist or a TRPV1 agonist.

20 13. The assay of one of claims 1 to 12, wherein the assay is an automated high throughput screening assay.

14. The assay of one of claims 1 to 13, wherein the assay is carried out using the patch-clamp technique.

25 15. Use of a screening assay of one of claims 1 to 14 for characterising the thermogenic properties of a TRPV1 modulator.

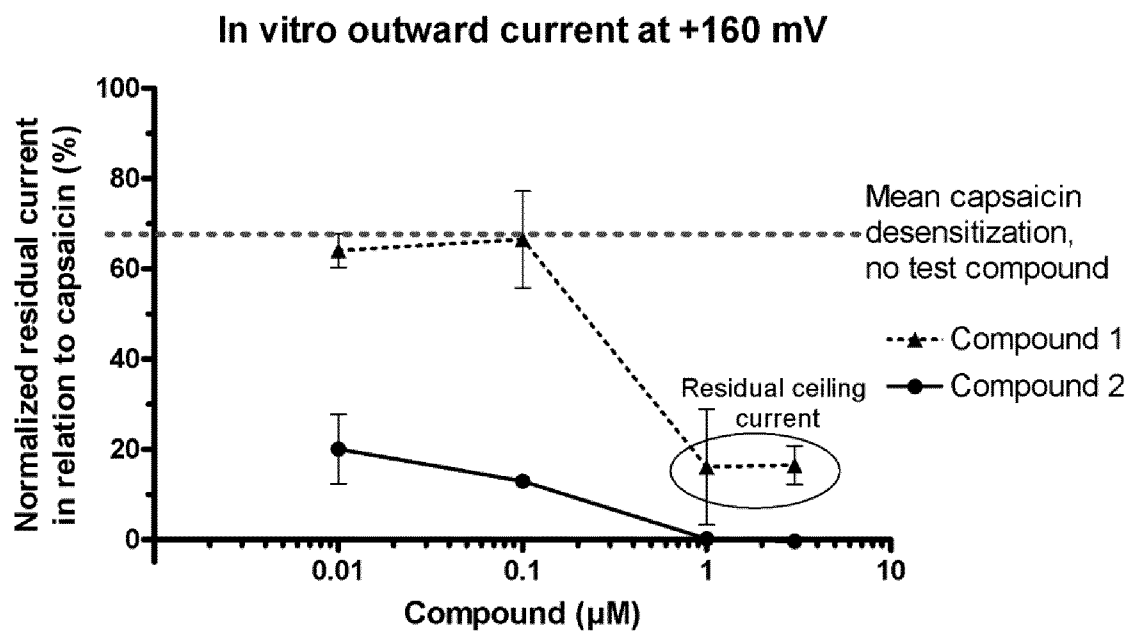


Figure 1

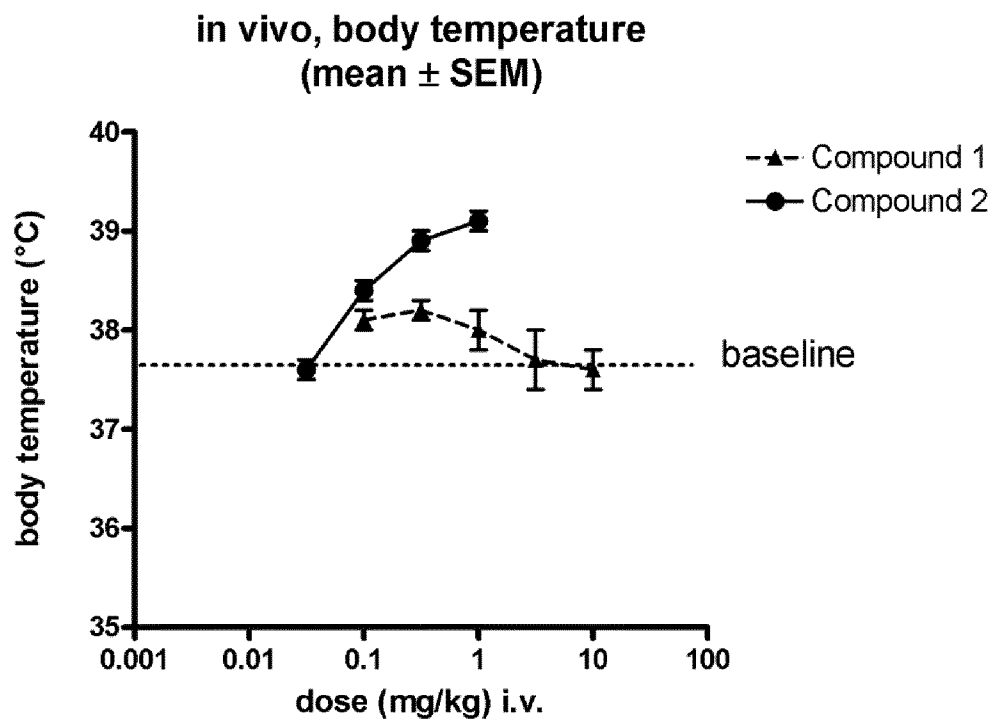


Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/025044

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50 G01N33/487 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

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

EPO-Internal, WPI Data, EMBASE, BIOSIS, COMPENDEX, INSPEC, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUEPER T ET AL: "Inhibition of TRPV1 for the treatment of sensitive skin", EXPERIMENTAL DERMATOLOGY ONLINE, WILEY-BLACKWELL PUBLISHING LTD, vol. 19, no. 11, 1 November 2010 (2010-11-01), pages 980-986, XP002692505, ISSN: 1600-0625, DOI: 10.1111/J.1600-0625.2010.01122.X [retrieved on 2010-07-07] page 981, column 1, paragraph 3; figure 1 ----- -/--	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 June 2016

Date of mailing of the international search report

07/07/2016

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/025044

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Reilly, R.M., et. al.: "Pharmacology of Modality-Specific Transient Receptor Potential Vanilloid-1 Antagonists That Do Not Alter Body Temperature", The Journal of Pharmacology and Experimental Therapeutics, vol. Vol. 342, No. 2 8 May 2012 (2012-05-08), pages 416-428, XP055225337, Retrieved from the Internet: URL:http://jpet.aspetjournals.org/content/342/2/416.full.pdf#page=1&view=FitH [retrieved on 2015-11-03] page 418, column 1, paragraph 5 - column 2, paragraph 1 page 421, column 2, paragraph 3 - page 424, column 1, paragraph 1; tables 2,5 -----	1-15
A	MIQUEL VIDAL-MOSQUERA ET AL: "Triazine-Based Vanilloid 1 Receptor Open Channel Blockers: Design, Synthesis, Evaluation, and SAR Analysis", JOURNAL OF MEDICINAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY, US , vol. 54, no. 21 10 October 2011 (2011-10-10), pages 7441-7452, XP002683215, ISSN: 0022-2623, DOI: 10.1021/JM200981S Retrieved from the Internet: URL:http://pubs.acs.org/doi/abs/10.1021/jm200981s [retrieved on 2011-09-27] page 7450, column 2, paragraph 7 - page 7451, column 1, paragraph 1 -----	1-15
A	WO 2005/087807 A1 (RENOVIS INC [US]; HACKOS DAVID [US]; SERAFINI TITO [US]; ORIKE NINA [U]) 22 September 2005 (2005-09-22) paragraphs [0179], [0186] - [0191] -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/025044

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005087807 A1	22-09-2005	US 2005266435 A1 WO 2005087807 A1	01-12-2005 22-09-2005
