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# TRPV1 antagonists that cause hypothermia, instead of hyperthermia, in rodents: Compounds' pharmacological profiles, in vivo targets, thermoeffectors recruited and implications for drug development

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## Abstract

**Aim:** Thermoregulatory side effects hinder the development of transient receptor potential vanilloid-1 (TRPV1) antagonists as new painkillers. While many antagonists cause hyperthermia, a well-studied effect, some cause hypothermia. The mechanisms of this hypothermia are unknown and were studied herein.

**Methods:** Two hypothermia-inducing TRPV1 antagonists, the newly synthesized A-1165901 and the known AMG7905, were used in physiological experiments in rats and mice. Their pharmacological profiles against rat TRPV1 were studied in vitro.

**Results:** Administered peripherally, A-1165901 caused hypothermia in rats by either triggering tail-skin vasodilation (at thermoneutrality) or inhibiting thermogenesis (in the cold). A-1165901-induced hypothermia did not occur in rats with desensitized (by an intraperitoneal dose of the TRPV1 agonist resiniferatoxin) sensory abdominal nerves. The hypothermic responses to A-1165901 and AMG7905 (administered intragastrically or intraperitoneally) were absent in *Trpv1*$^{-/-}$ mice, even though both compounds evoked pronounced hypothermia in *Trpv1*$^{+/+}$ mice. In vitro, both A-1165901 and AMG7905 potently potentiated TRPV1 activation by protons, while potently blocking channel activation by capsaicin.

**Conclusion:** TRPV1 antagonists cause hypothermia by an on-target action: on TRPV1 channels on abdominal sensory nerves. These channels are tonically activated by protons and drive the reflectory inhibition of thermogenesis and tail-skin vasoconstriction. Those TRPV1 antagonists that cause hypothermia further inhibit these cold defences, thus decreasing body temperature.

**Significance:** TRPV1 antagonists (of capsaicin activation) are highly unusual in that they can cause both hyper- and hypothermia by modulating the same mechanism. For drug development, this means that both side effects can be dealt with simultaneously, by minimizing these compounds' interference with TRPV1 activation by protons.

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**KEYWORDS**
hyperthermia, hypothermia, protons, thermoregulation, TRPV1 antagonists, vanilloids

## 1 | INTRODUCTION

Antagonists of the transient receptor potential vanilloid-1 (TRPV1) channel are widely viewed as candidates for becoming novel non-opioid analgesics. Reflecting this trend, numerous selective and potent TRPV1 antagonists were synthesized by many pharmaceutical companies and swiftly moved to clinical trials at the onset of the 21st century (reviewed in refs. 1-3). Almost immediately, several of them were reported to cause hyperthermia in laboratory animals[4-7] and later in humans.[8-12] This hyperthermia was shown to be an on-target side effect, ie to occur through the TRPV1 channel.[6,13-15] It appeared that TRPV1 antagonists cause hyperthermia by blocking the tonic suppression of the autonomic cold defences: thermogenesis and skin vasoconstriction.[6,8] This tonic suppression occurs through non-thermal activation of TRPV1 channels somewhere in the abdomen, perhaps in the viscera or muscles.[6] Later, it was discovered that the hyperthermia was caused only by antagonists that potently blocked the proton (low pH) activation mode of TRPV1 channels (typically, polymodal antagonists, aka the first-generation antagonists). The second-generation, modality-selective antagonists, that is those that did not block the proton activation mode (or only partially blocked it), did not cause hyperthermia.[13,16] The potency of an antagonist to block the thermal (heat) mode of TRPV1 activation appeared to be unrelated to its ability to cause hyperthermia, whereas the potency to block the vanilloid (capsaicin) mode of activation was shown to make either some or no contribution to the hyperthermic effect.[13] Overall, a scenario has emerged, in which TRPV1 antagonists that are potent blockers of TRPV1 activation by protons act on TRPV1 channels that are tonically activated (presumably, by a low pH somewhere in the abdomen) and block this tonic activation, and that this blockade disinhibits autonomic cold defences, thus resulting in hyperthermia.[13,17,18]

The picture became more complex after several TRPV1 antagonists were found to decrease the deep body temperature ($T_b$), that is cause hypothermia. The list of hypothermia-inducing compounds includes several small-molecule antagonists, viz., 5′-iodo-resiniferatoxin (5′-I-RTX),[19,20] Amgen's AMG7905 and AMG8562,[16] Abbott Laboratories' A-425619,[21] AbbVie's Compound 3[22] and Schwarz Pharma's JYL1421,[13] as well as at least 1 polypeptide antagonist, APHC3.[23] In agreement with the fact that TRPV1 agonists also cause hypothermia,[17,24,25] several hypothermia-causing TRPV1 antagonists, viz., 5′-I-RTX[20,26] and Compound 3,[22] were found to be partial agonists, whereas the partial antagonist APHC3 was found to potentiate the effects of low concentrations of capsaicin.[27] However, several other hypothermia-inducing TRPV1 antagonists, viz., A-425619,[28] AMG7905 and AMG8562[16] and JYL1421,[5] showed no TRPV1 agonistic or capsaicin-potentiating activity. While a few, largely hypothetical, mechanisms of the hypothermic effect of TRPV1 antagonists have been proposed,[13,21] there has been no systematic study focused on the hypothermic effect of TRPV1 antagonists.

Many classes of biologically active substances, and even individual substances, have a potential to both increase and decrease $T_b$. For example, the same dose of the psychostimulant 3,4-methylenedioxymethamphetamine,[29,30] bioactive phospholipid platelet-activating factor[31,32] or bacterial lipopolysaccharide (LPS)[33] can either increase or decrease $T_b$ in the same species, depending on the ambient temperature ($T_a$). In the case of LPS, its opposite effects on $T_b$ have been shown to be mediated by different mechanisms, that is to involve different receptors,[34] different enzymes,[35] different brain structures[36] and, at least in some cases, different thermoeffectors.[37] Another example of a substance with a dual (hyper- and hypothermic) thermoregulatory action is cholecystokinin (CCK) octapeptide. CCK induces hyperthermia in rats by acting on $CCK_B$ receptors in the brain, but hypothermia by acting on $CCK_A$ receptors in the periphery.[38,39] Tryptophan also can cause both hyperthermia (by acting on serotonin receptors 5-$HT_2$) and hypothermia (by acting on 5-$HT_{1A}$ receptors).[40] Intrathecal injections of noradrenaline can cause hypothermia (via an action in the spinal cord) and hyperthermia (via a peripheral action).[41] Similarly, intracerebroventricular injections of noradrenaline cause hypothermia (via a central action) and hyperthermia (as the drug leaks from the brain and acts in the periphery).[42] Intrabrain microinjections of clonidine were also reported to cause both hypo- and hyperthermia; in that case, hypothermia was an authentic, $\alpha_2$-adrenoreceptor-mediated effect of the drug, whereas hyperthermia was an artefact caused by the local release of prostaglandins due to brain tissue damage associated with the injection.[43]

These examples demonstrate that compounds that cause both hyper- and hypothermia typically do so by acting on totally different mechanisms. The 2 effects often occur under different conditions (eg different $T_a$), originate at different locations, are triggered from different receptors and

otherwise involve different pharmacological and physiological mechanisms. It can be expected, therefore, that TRPV1 antagonists also cause their hyper- and hypothermic effects via independent actions. Hence, the hypothermic side effect of TRPV1 antagonists introduces a new concern for drug development. The known hyperthermic side effect is widely acknowledged as a major problem for the development of TRPV1 antagonists.[44,45] Does the hypothermic effect, with its unknown mechanisms, represent another major problem?

In the present work, we report the synthesis of A-1165901, a urea-type selective and potent TRPV1 antagonist, and show that it causes hypothermia in rats and mice. We use A-1165901, as well as AMG7905, the antagonist for which the hypothermic effect had already been demonstrated in rats by Lehto et al[16] to reveal their in vitro pharmacological profiles and the physiological mechanisms of the hypothermic response. We specifically test whether the hypothermic response affects mechanisms known to be involved in the hyperthermic response to TRPV1 antagonists. We believe our results clarify the nature of the hypothermic side effect of TRPV1 antagonists and, as such, are highly instrumental for their further development.

## 2 | RESULTS

### 2.1 | A-1165901 decreases deep $T_b$ in rats

First, we screened A-1165901 for an effect on deep $T_b$ in rats. A-1165901 or its vehicle was administered by gavage in the telemetry set-up. Vehicle administration in this set-up is known to cause stress hyperthermia. Indeed, the deep $T_b$ (abdominal) rapidly increased by 0.5-1.3°C and returned to baseline at 135 minutes post-administration (Figure 1; $P < .05$). Compared to vehicle, A-1165901 (41 mg kg$^{-1}$, intragastrically, i.g.) caused a marked drop in $T_b$, with the biggest intergroup difference of 1.6°C at 60 minutes ($P < .001$). The effects of both the treatment (ANOVA, $F_{(1,202)} = 70.9$, $P < .001$) and time ($F_{(16,202)} = 9.1$, $P < .001$) were significant, and so was the treatment × time interaction ($F_{(16,202)} = 7.7$, $P < .001$). A significant $T_b$ difference between the treatments occurred at 45-135 minutes post-administration (Fisher's LSD test, $P < .01$). Importantly, in the A-1165901-treated rats, $T_b$ decreased below its basal level and stayed there for the period 60-130 minutes post-administration (Fisher's LSD test, $P < .05$). These results confirm the bioavailability of A-1165901 and show that it decreases deep $T_b$.

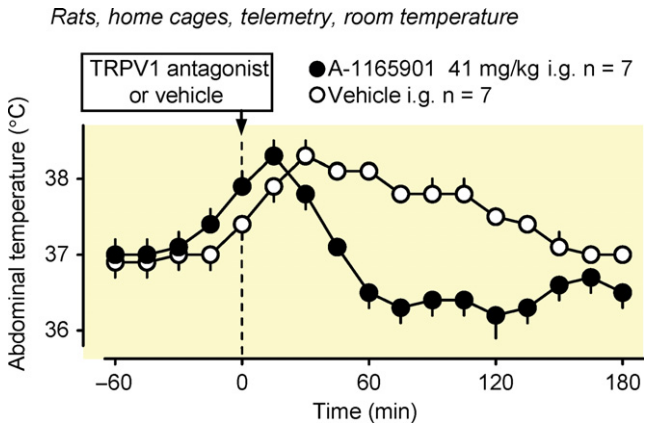


**FIGURE 1** The i.g. administration of A-1165901 by gavage to rats causes a decrease in the abdominal $T_b$. Here and in Figures 2-5, n is the number of animals in each experimental group

### 2.2 | A-1165901-induced hypothermia involves tail-skin vasodilation at thermoneutrality but inhibition of thermogenesis in the cold

To characterize the hypothermic effect of A-1165901 more precisely, we administered this compound in a stress-free fashion and studied its effects on deep $T_b$ and thermoeffector mechanisms under different thermal conditions. A-1165901 or its vehicle was infused through a pre-implanted intraperitoneal (i.p.) catheter; the infusions were performed from outside the chamber, without disturbing the rats. The experiments were conducted in the respirometry set-up at either 26°C (the lower end of the thermoneutral zone for rats in this set-up) or a subneutral $T_a$ of 17°C (in the cold). At either $T_a$, A-1165901 (3 mg kg$^{-1}$, i.p.) decreased deep $T_b$ by ~1.0°C compared to vehicle, with the nadirs at 50-70 minutes (Figure 2). The treatment × time interaction was significantly different between A-1165901 and vehicle treatment (ANOVA, $F_{(24,375)} = 2.9$, $P < .001$, and $F_{(24,200)} = 1.1$, $P < .001$, at 26 and 17°C respectively). At 26°C, all rats exhibited mild tail-skin vasodilation and relatively low thermogenesis before administration of the drug or its vehicle (Figure 2A). A-1165901 induced prompt elevation of heat loss at this $T_a$ (ANOVA, $F_{(24,375)} = 4.0$, $P < .001$). The heat loss index (*HLI*) was significantly higher in the A-1165901-treated than vehicle-treated rats at 10-60 minutes post-administration (Fisher's LSD test, $P < .05$). Although the rate of oxygen consumption ($\dot{V}O_2$) tended to be lower in A-1165901-treated rats, as compared to controls, the difference never reached the level of significance at this $T_a$. In contrast, at 17°C, the rats' tails were strongly vasoconstricted throughout the experiment, and the pre-treatment $\dot{V}O_2$ level was elevated compared to the thermoneutral conditions, thus indicating the presence of cold-induced thermogenesis (Figure 2B). Administration of A-1165901 did not cause tail-skin vasodilation at this $T_a$, but it strongly reduced thermogenesis, as compared to controls (ANOVA, $F_{(1,200)} = 4.8$, $P < .05$). These results demonstrate that non-stressful administration of A-1165901

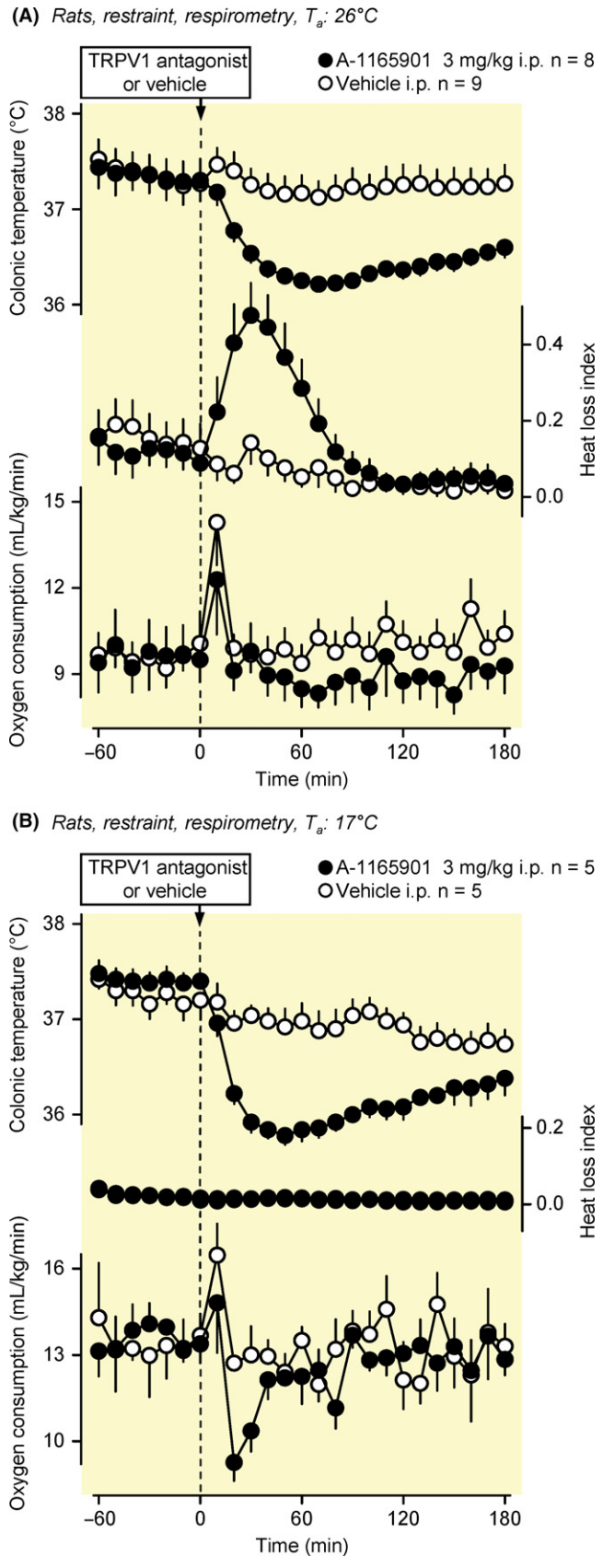


**FIGURE 2** A-1165901-induced hypothermia is brought about by either tail-skin vasodilation or inhibition of thermogenesis. (A) At a $T_a$ of 26°C (the low end of the thermoneutral zone), A-1165901 produces hypothermia, which is accompanied by an increased *HLI* (an indicator of tail-skin vasodilation) with only minimal or no effect on $VO_2$ (indicator of non-shivering thermogenesis). (B) In the cold (17°C), A-1165901 causes hypothermia through a decrease in $VO_2$, without affecting cold-induced tail-skin vasoconstriction

to rats produces hypothermia, which, depending on $T_a$, occurs due to either tail-skin vasodilation (at thermoneutrality) or inhibition of thermogenesis (in the cold).

## 2.3 | A-1165901 causes hypothermia by acting on intra-abdominal targets

To test whether the hypothermic effect of A-1165901 is triggered from the sensory nerves in the abdomen, we induced localized intra-abdominal TRPV1 desensitization with a low dose of RTX (20 μg $kg^{-1}$, i.p.) and, 10-13 days later, studied the thermoregulatory response of the desensitized rats to A-1165901 in the thermocouple set-up at a slightly subneutral $T_a$ of 27°C. Administration of A-1165901 (3 mg $kg^{-1}$, i.p.) to vehicle-pre-treated rats resulted in a pronounced drop of deep $T_b$ and an increase in the *HLI*, as expected. The $T_b$ response was strongly attenuated in the desensitized rats, and no change in the *HLI* occurred (Figure 3A). The effects of A-1165901 were significantly different between the RTX- and vehicle-pre-treated groups with regard to both $T_b$ and the *HLI* (ANOVA, $F_{(1,275)} = 86.0$, $P < .001$, and $F_{(1,275)} = 11.9$, $P < .001$ respectively). In RTX-pre-treated rats, the attenuation of the $T_b$ decrease was significant at 20-120 minutes, and their *HLI* was lower at 20-40 minutes, as compared with controls (Fisher's LSD test, $P < .05$). These data indicate that the hypothermic response to TRPV1 antagonists examined to date is mediated by intra-abdominal sensory nerves.

To confirm the desensitization of intra-abdominal TRPV1 channels, we studied the writhing response to RTX (0.1 μg $kg^{-1}$, i.p.). This response was nearly completely ablated in desensitized rats compared to sham-desensitized rats ($t_{(13)} = 5.7$, $P < .001$; Figure 3B). To confirm that the desensitization did not spread throughout the body, we studied the eye-wiping response to topical RTX; no meaningful difference was found in the sensitivity of corneal TRPV1 channels between desensitized and sham-desensitized rats (Figure 3B). In either the writhing test or eye-wiping test, the responses to vehicle did not differ between RTX-desensitized and sham-desensitized rats (data not shown). In accordance with earlier studies,[6,19] these results confirm that the function of TRPV1 channels was impaired solely in the abdominal cavity of RTX-desensitized rats.

## 2.4 | TRPV1 antagonist–induced hypothermia is mediated by TRPV1 channels

Next, we used mice with ($Trpv1^{-/-}$) or without ($Trpv1^{+/+}$) a homozygous mutation in the *Trpv1* gene to determine

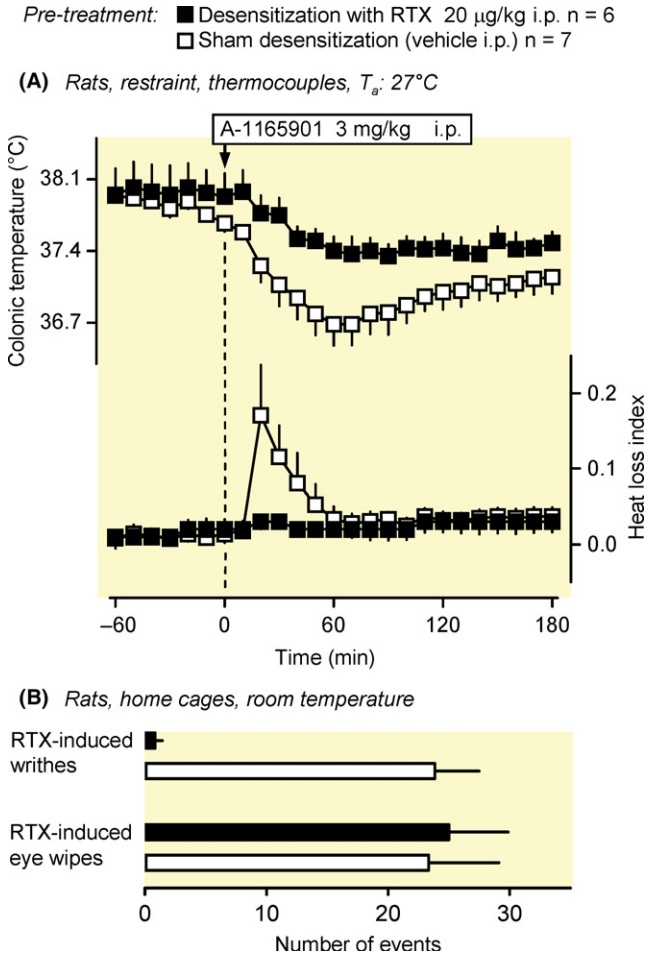


**FIGURE 3** A-1165901-induced hypothermia is abolished in rats desensitized with a prior i.p. injection of RTX. (A) The hypothermic effect of A-1165901 is present in rats pre-treated with the vehicle (sham desensitization), but it is absent in rats pre-treated with RTX to induce localized intra-abdominal TRPV1 desensitization. (B) In rats with sham desensitization, TRPV1 channels can be activated by RTX from both within and outside the abdominal cavity (the writhing and eye-wiping reflexes respectively). In rats with RTX desensitization, only the function of extra-abdominal TRPV1 channels is intact, while the function of intra-abdominal TRPV1 channels is eliminated

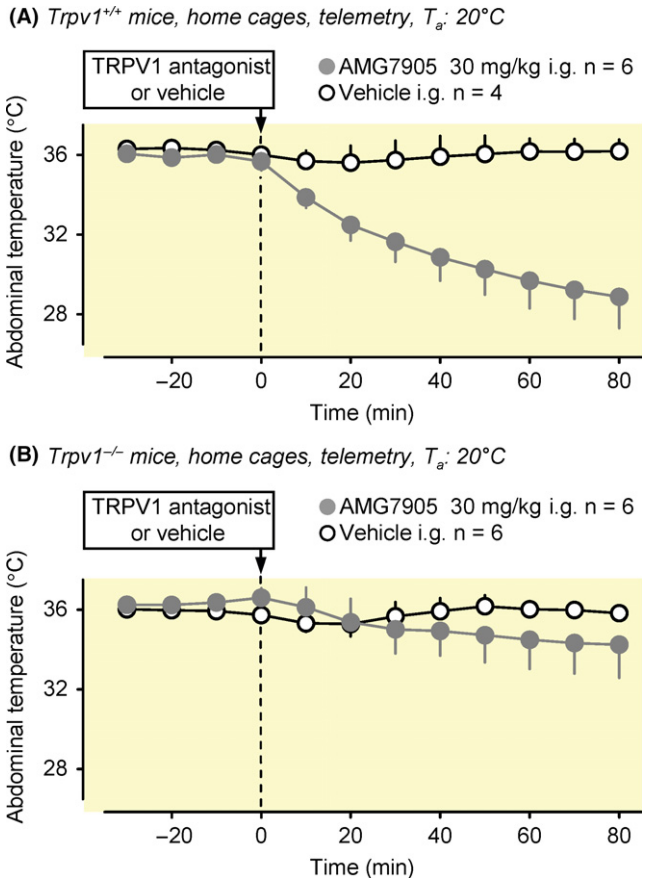


**FIGURE 4** AMG7905-induced hypothermia is mediated by TRPV1 channels. (A) The i.g. administration of AMG7905, but not vehicle, produces hypothermia in $Trpv1^{+/+}$ mice. (B) The AMG7905-induced hypothermia is absent in $Trpv1^{-/-}$ mice

whether the TRPV1 antagonist-induced hypothermia is an on-target effect. In the first experiment aimed at addressing this goal, we administered AMG7905 by gavage. As the expected result was hypothermia, these experiments were conducted at a slightly subneutral $T_a$. $Trpv1^{+/+}$ mice responded to the i.g. administration of AMG7905 with pronounced hypothermia (compared to vehicle), with the biggest intertreatment difference of 7.8°C at 140 minutes (Fisher's LSD test, $P < .001$; Figure 4A). In contrast, AMG7905 did not cause hypothermia in $Trpv1^{-/-}$ mice (Figure 4B).

In the second experiment, we administered either A-1165901 or AMG7905 through a pre-implanted i.p. catheter in a non-stressful manner. In $Trpv1^{+/+}$ mice, both compounds caused a pronounced drop in $T_b$ (>2°C), as compared to the vehicle (ANOVA, $P < .001$ for both compounds; Figure 5A). In contrast, neither A-1165901 nor AMG7905 had any effect on $T_b$ in $Trpv1^{-/-}$ mice (Figure 5B). This study shows that both TRPV1 antagonists used caused hypothermia by acting on TRPV1 channels.

## 2.5 | A-1165901 and AMG7905 block the activation of the TRPV1 channel by capsaicin, but potentiate its activation by protons in vitro

Because hyperthermia-inducing TRPV1 antagonists have a high potency in blocking the proton mode of TRPV1 activation,[13,16] we studied the effects of hypothermia-inducing TRPV1 antagonists on this mode. We also studied their effects on TRPV1 activation by vanilloids (capsaicin), because a compound has to block this activation mode in order to be considered a TRPV1 antagonist.

First, we studied the effects of A-1165901 on rat and human TRPV1 channels (expressed on recombinant HEK293 cells) in a fluorometric imaging plate reader (FLIPR)-based screening system, which is routinely used in

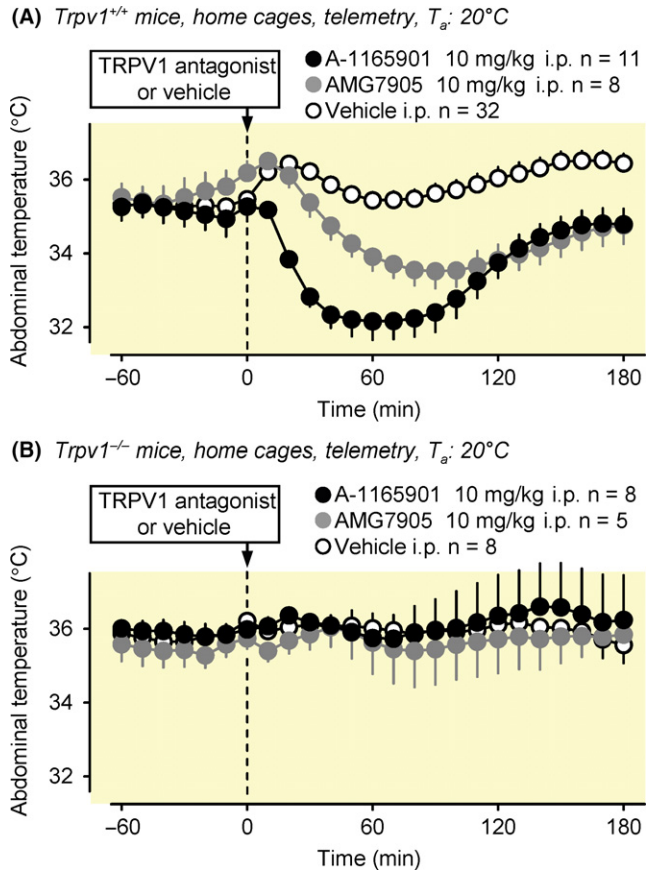


**FIGURE 5** TRPV1 antagonist–induced hypothermia is mediated by TRPV1 channels. (A) The i.p. administration of A-1165901 or AMG7905, but not vehicle, produces hypothermia in *Trpv1*$^{+/+}$ mice. (B) The hypothermic response to either A-1165901 or AMG7905 is abolished in *Trpv1*$^{-/-}$ mice

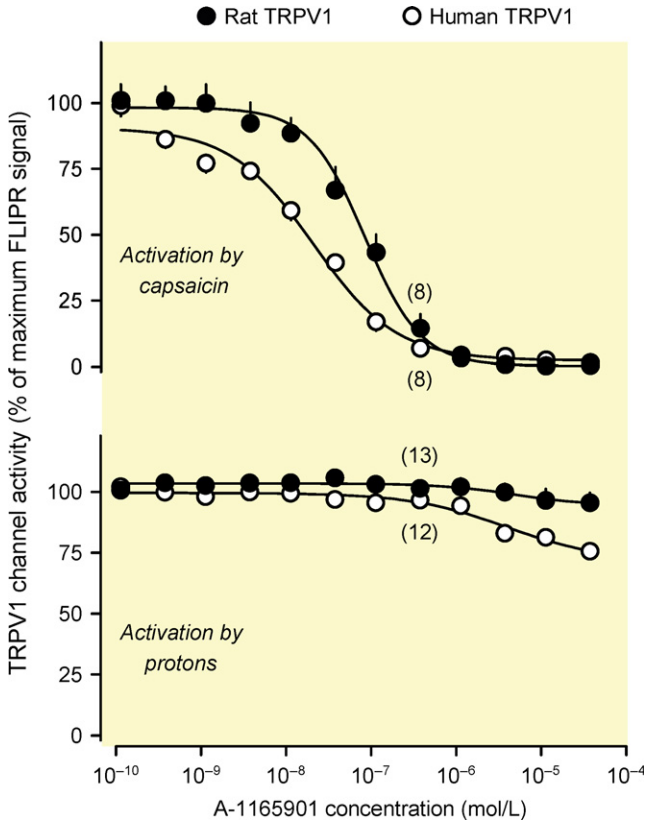


**FIGURE 6** In a total intracellular $Ca^{2+}$ assay using a FLIPR-based system, A-1165901 blocks the activation of rat and human TRPV1 channels by capsaicin in a concentration-dependent manner, but has no meaningful effect of their activation by protons. This assay cannot reveal any potentiating effect on TRPV1 activation. Here and in Figure 7, the numbers in parentheses are the numbers of replications used in each experiment

many laboratories, including one of ours, to assay the total intracellular $Ca^{2+}$ concentration.[18,28,46] A-1165901 completely blocked the capsaicin-induced increase in intracellular $Ca^{2+}$ in cells expressing either rat or human TRPV1 channels ($IC_{50}$ values of 79.7 ± 6.8 and 19.0 ± 3.1 nmol $L^{-1}$ respectively; Figure 6). A-1165901 also blocked the activation of rat and human TRPV1 by *N*-arachidonoyl-dopamine (NADA), an endogenous agonist ($IC_{50}$ of 112 and 7 nmol $L^{-1}$ respectively; data not shown). However, even at the highest concentration used (11.25 μmol $L^{-1}$), A-1165901 did not fully block the proton-induced activation of either the rat or human TRPV1 channel. In this activation mode, A-1165901 exhibited only partial inhibition (17.7 ± 3.7% at the rat and 23.7 ± 7.4% at the human TRPV1 channel) with $IC_{50}$ > 37.5 μM for both species (Figure 6). Importantly, A-1165901 did not have any partial agonistic activity, as no effect on the total intracellular $Ca^{2+}$ concentration was observed upon its addition to the cell media in the absence of capsaicin, NADA or acid. A-1165901 was selective for TRPV1, as its potency in blocking other TRP channels was several orders of magnitude lower, eg $IC_{50}$ > 25 μmol $L^{-1}$ for both TRP ankyrin-1 (activated by 30 μmol $L^{-1}$ isothiocyanate) and TRP melastatin-8 (activated by 10 μmol $L^{-1}$ menthol; data not shown). These findings confirm that A-1165901 potently and selectively blocks the vanilloid activation mode in both rat and human TRPV1 channels. They also show that A-1165901 does not block the proton-induced activation of this channel in either species. However, the FLIPR-based measurements of the total intracellular $Ca^{2+}$ concentration did not clarify whether hypothermia-inducing antagonists potentiate the activation of TRPV1 by protons, as proposed earlier,[16] as any significant intracellular $Ca^{2+}$ release can mask changes in the $Ca^{2+}$ influx.

We then studied the effects of A-1165901 and AMG7905 on the $^{45}Ca^{2+}$ uptake directly, with a scintillation counter. This set-up has been successfully utilized in our previous studies to detect the potentiation of TRPV1 activation in different modes by a variety of TRPV1 antagonists.[5,16,47] In this set-up, both A-1165901 and AMG7905 potently blocked activation of TRPV1 by capsaicin in a concentration-dependent manner (Figure 7), with $IC_{50}$ values of 3.7 ± 1.4 and 29.3 ± 12.4 nmol $L^{-1}$ respectively.

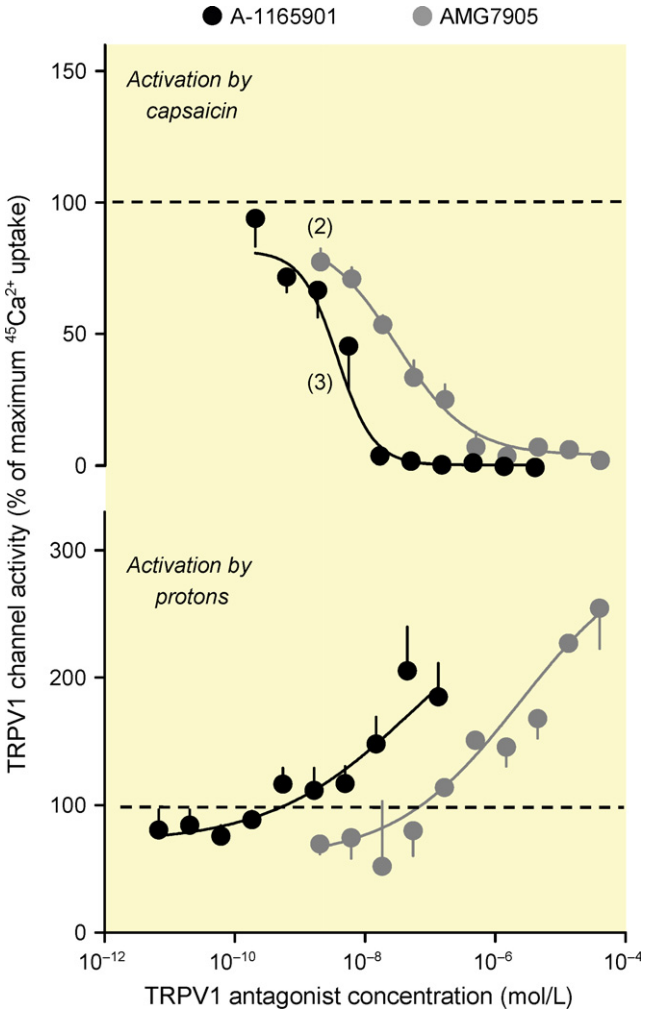


**FIGURE 7** In a $^{45}Ca^{2+}$ uptake assay using a scintillation counter, A-1165901 and AMG7905 cause concentration-dependent blockade of the activation of the TRPV1 channel by capsaicin, while both compounds strongly potentiate the activation of the channel by protons

In the proton activation mode, both compounds produced concentration-dependent potentiation of $^{45}Ca^{2+}$ influx. For A-1165901, this effect was observed at concentrations as low as <1 nmol $L^{-1}$. Hence, both hypothermia-inducing TRPV1 antagonists tested, viz., A-1165901 and AMG7905, appeared to be potent blockers of the capsaicin mode and potent potentiators of the proton mode of TRPV1 activation.

## 3 | DISCUSSION

### 3.1 | The hypothermic effect of TRPV1 antagonists

We describe the synthesis of A-1165901, a novel TRPV1 antagonist of the urea chemotype. We show that it causes not hyperthermia, the effect that has been reported for most TRPV1 antagonists, but hypothermia, the effect that is characteristic of only a few antagonists (reviewed in ref. 17). A-1165901 decreases deep $T_b$ in rats upon either i.g. or i.p. administration, and even more so in mice upon i.p. administration. We then characterize the hypothermic effect of A-1165901.

We show that depending on the $T_a$, either the tail-skin vasculature or thermogenesis can be recruited in the hypothermic response to A-1165901 in rats. The tail-skin vasodilation is the predominant effector in a thermoneutral environment, whereas the inhibition of thermogenesis is the sole autonomic effector of the hypothermic response to A-1165901 in the cold. In this respect, the hypothermic response to this compound is similar to the hyperthermic responses to TRPV1 antagonists, viz., AMG0347[6] and AMG 517.[8,48] AMG0347 and AMG 517 increase deep $T_b$ in rats by affecting the same thermoeffectors (but in the opposite direction): the tail-skin vasculature becomes constricted, and thermogenesis is activated. In the case of hyperthermic antagonists, the action on the vasomotion also plays a major role in a neutral or warm environment, whereas the action on thermogenesis is the major mechanism under the conditions of cold exposure.[6,8,48] Hence, the hypo- and hyperthermic responses to TRPV1 antagonists use the same autonomic thermoeffectors, and the way these effectors are recruited in the hypo- and hyperthermic responses shows the same dependence on the $T_a$.

We also show that A-1165901-induced hypothermia does not occur in rats previously treated with a low i.p. dose of RTX (20 μg $kg^{-1}$). This RTX treatment desensitizes the sensory nerves in the abdomen, but not in the thoracic viscera, brain, cornea or skin,[6,19] and this desensitization prevents the development of hyperthermia in response to AMG0347[6] or A-889425,[49] and also in response to AMG 517 or AMG8163 (A. Garami, A. A. Steiner, and A. A. Romanovsky, unpublished observations). In other words, both the hypo- and hyperthermic responses to TRPV1 antagonists are triggered from the same location: the abdomen, perhaps the intra-abdominal viscera or abdominal-wall muscles.

Prior to this study, it was not established whether the hypothermic effect of TRPV1 antagonists is an on-target effect (ie whether it occurs via TRPV1 channels). In fact, several authors hypothesized that hypothermia is not an on-target effect.[13,21] By the same token, there are reports showing that selective TRPV1 antagonists of different chemotypes cause hypothermia,[13,16,19-23] which is suggestive of an on-target nature of the hypothermic effect of TRPV1 antagonists. In this study, we show definitively that the hypothermic effects of A-1165901 and AMG7905 occur only in the presence of TRPV1 channels. Indeed, neither of the 2 TRPV1 antagonists studied decreases deep

$T_b$ in *Trpv1*$^{-/-}$ mice upon the i.p. administration, even though both compounds evoke a pronounced hypothermic response in *Trpv1*$^{+/+}$ mice. Furthermore, we also show that the i.g. administration of AMG7905 does not cause hypothermia in *Trpv1*$^{-/-}$ mice, while causing pronounced hypothermia in wild-type mice. For comparison, the on-target nature of the hyperthermic effect has been demonstrated using *Trpv1*$^{-/-}$ mice for several TRPV1 antagonists, viz., AMG0347, AMG 517 and AMG9810.[6,13-15] In addition, AMG 517 has also been shown to prevent ketamine-induced hypothermia in mice by acting, at least partially, on TRPV1 channels.[48]

We further report that both hypothermia-inducing TRPV1 antagonists studied (A-1165901 and AMG7905) potently potentiate the activation of TRPV1 channels by protons in vitro, while potently blocking the capsaicin mode. In view of the pharmacological complexity of TRPV1,[26,45,50] it is important to note that neither AMG7905 nor A-1165901 shows any agonistic activity (ie does not activate the TRPV1 channel by itself, in the absence of any agonists). These new data are in line with the report by Lehto et al[16] who first observed that AMG7905 and AMG8562 potentiated TRPV1 activation by protons in vitro and proposed that such potentiation correlated with the ability of a TRPV1 antagonist to cause hypothermia. Hence, whereas the TRPV1 antagonists that block the proton mode of TRPV1 activation are now firmly established to cause hyperthermia,[13,16,18] it is also becoming clear that the compounds that potentiate this mode cause hypothermia.

This study also demonstrates that measurements of the total intracellular $Ca^{2+}$ concentration (eg by FLIPR) are poorly suited for the identification of the pharmacological profile of hypothermia-inducing antagonists, as they often fail to reveal a potentiating effect due to the fact that any significant intracellular $Ca^{2+}$ release can readily mask changes in the $Ca^{2+}$ influx. Direct measurements of the $Ca^{2+}$ influx (eg with a scintillation counter) should be used instead.

## 3.2 | The hypo- and hyperthermic effects of TRPV1 antagonists occur through an inverse modulation of the same pathway: a unifying concept

When a compound can cause either hypo- or hyperthermia, it usually produces the 2 effects by acting on 2 different mechanisms (see Introduction). A mechanism that causes hyperthermia cannot cause hypothermia: it either increases $T_b$ (when active) or produces no effect (when inactive). The opposite is also true: a mechanism that causes hypothermia cannot increase $T_b$. The results reported here show that TRPV1 antagonists can act on a highly unusual, possibly unique mechanism, which can be modulated in opposite directions to trigger the opposite changes of $T_b$. The hyperthermia-inducing TRPV1 antagonists are potent blockers of the proton mode of TRPV1 activation (Figure 8A). They cause hyperthermia by acting on TRPV1 channels (an on-target effect) at an unidentified location in the abdomen and blocking the tonic activation of these channels by protons. This blockade results in the disinhibition of autonomic cold defences (thermogenesis and tail-skin vasoconstriction in rodents), thus leading to hyperthermia. The so-called thermally neutral TRPV1 antagonists (ie those that do not affect deep $T_b$) do not have an effect on the proton mode of TRPV1 activation (Figure 8B) and do not affect the thermoregulatory reflexes from the abdominal TRPV1 channels. The hypothermia-inducing TRPV1 antagonists are potentiators—not blockers—of the proton mode (Figure 8C). They act on the abdominal TRPV1 channels to enhance the tonic activation by protons, further strengthening the tonic inhibition of autonomic cold defences, which leads to hypothermia. This unifying concept explains all possible effects of TRPV1 antagonists on deep $T_b$.

## 3.3 | An important corollary

This study also clarifies the important question related to the pharmacological profile of the hyperthermia-inducing TRPV1 antagonists: Do they possess a high potency of blocking the vanilloid (capsaicin) mode of TRPV1 activation, in addition to the high potency of blocking the proton mode? In the past, we attempted to answer this question using a mathematical model and analysing the profiles of 7 TRPV1 antagonists that caused hyperthermia and 1 antagonist (JYL1421) that caused hypothermia at high doses.[13] At that time, we did not know whether the hypo- and hyperthermic effects of TRPV1 antagonists had distinct mechanisms or, alternatively, stemmed from the opposite modulation of the same mechanism. Hence, we ran our model on 2 different sets of data: including and excluding the results obtained with the hypothermia-inducing compound JYL1421. Both analyses showed that the potency of blocking the proton mode of TRPV1 activation was very important for the ability of an antagonist to cause the hyperthermic effect and that the potency to block the temperature mode of TRPV1 activation was irrelevant. Regarding the capsaicin mode of activation, the 2 analyses produced different results. Without the JYL1421 data, the model showed that the potency of an antagonist to block the capsaicin mode also (in addition to its potency to block the proton mode) contributed to the ability to cause hyperthermia. With the JYL1421 data, the analysis showed that the potency of blocking the capsaicin mode was irrelevant. Based on the present study with A-1165901 and AMG7905, we now know that the hypothermic effect of TRPV1 antagonists involves the same mechanism as the

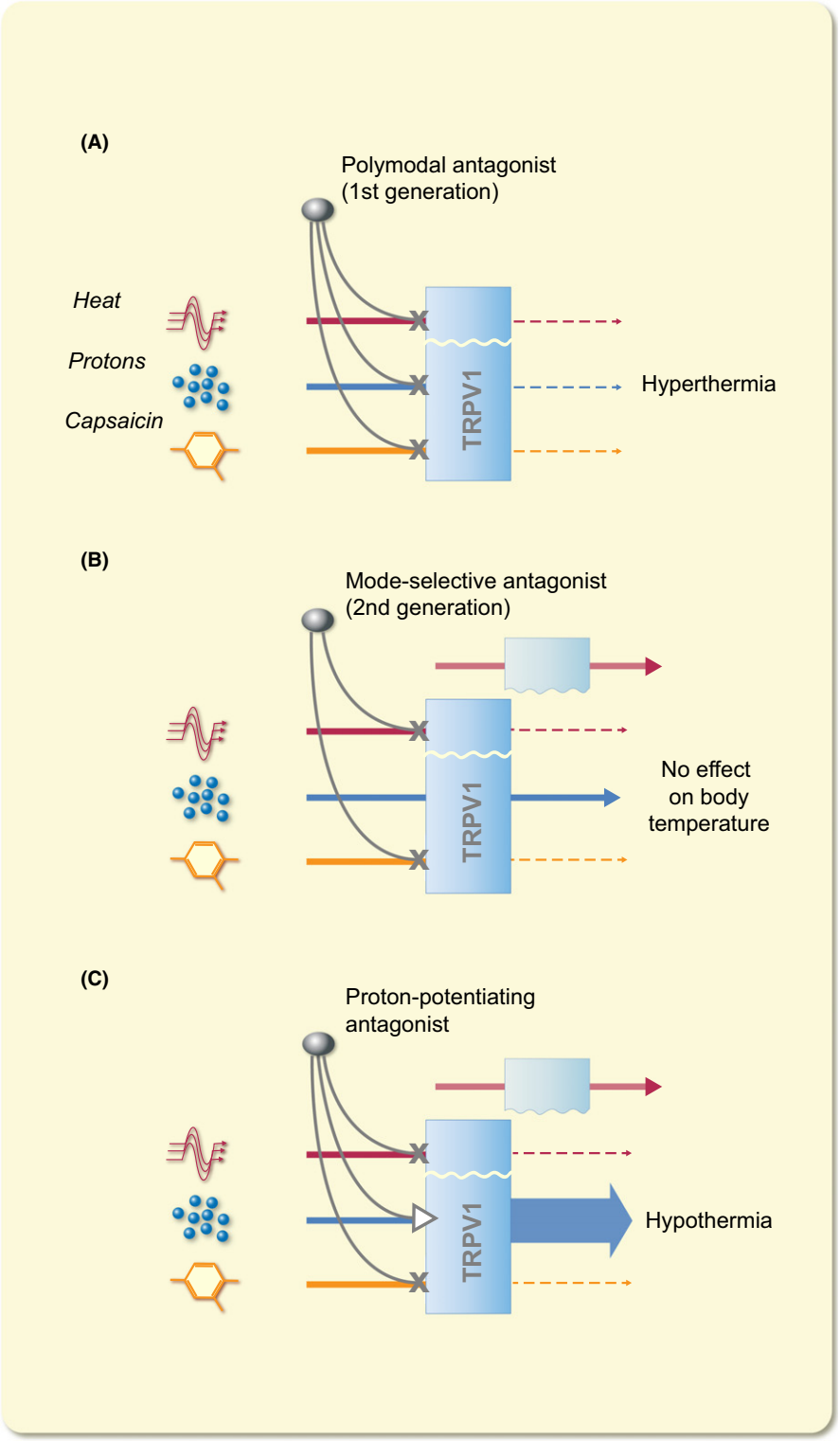


**FIGURE 8** Thermal effects of TRPV1 antagonists are determined by their action on the proton mode of TRPV1 activation. (A) Designed as broad-spectrum analgesics, polymodal TRPV1 antagonists potently block all 3 modes of TRPV1 activation, including the proton mode. They cause hyperthermia. (B) Modality-selective TRPV1 antagonists were designed in an effort to create thermally neutral antagonists. They have no potent effect on the proton activation mode, and they have no effect on deep $T_b$. As all TRPV1 antagonists, they potently block the capsaicin activation mode, and some of them are also potent blockers of the heat mode (eg capsazepine against rat TRPV1[13]), whereas others are not (eg NEO6860 against human TRPV1[53]). (C) A new functional class of TRPV1 antagonists was discovered as a by-product of the search for thermally neutral antagonists. Compounds of this new class potentiate the proton mode of TRPV1 activation and cause hypothermia

hyperthermic effect. Therefore, the JYL1421 data needed to be included in the mathematical model. Hence, the pharmacological profile of TRPV1 antagonists that cause hyperthermia requires a high potency in blocking the proton mode of TRPV1 activation and does not depend at all on the compound's potency to block either the temperature mode or the capsaicin mode.

### 3.4 | Thermally neutral TRPV1 antagonists: implications for drug development

Based on the current results and the retrospective analysis of our earlier results,[13] the following pharmacological profile of an "ideal" thermally neutral TRPV1 antagonist emerges. To cause no effects on $T_b$, this antagonist should neither block

nor potentiate the proton mode of TRPV1 activation, but to remain an overall potent blocker of the channel, it should be highly potent in blocking both the capsaicin and temperature modes. The latter 2 modes are not related to the hyper- and hypothermic side effects (Figure 8).

All thermally neutral TRPV1 antagonists reported so far[13,16,51-53] seem to fit this scenario. Nilius and Szallasi[54] point at 2 compounds, PHE377 and JTS-653, as potential exceptions, but do not support their claim with data. The polymodal antagonist JTS-653 has been reported to potently block proton-induced activation of both rat and human TRPV1 channels and to cause hyperthermia in rats;[55] therefore, it fully fits the proposed model. As for PHE377, no data regarding this compound have been published in the peer-reviewed literature. Yet another compound, V116517, while potently blocking TRPV1 activation by protons and causing hyperthermia in rats,[56] caused no hyperthermia in a clinical trial in humans.[57] However, at doses used, V116517 showed no or low efficacy in some capsaicin pain tests in this trial, while being a slightly more potent blocker of human TRPV1 activation by capsaicin than by protons. Hence, it is possible that the doses of V116517 used were insufficient to block the proton mode and cause hyperthermia. Furthermore, no data on $T_b$ were presented in the report whatsoever,[57] thus leaving the authors' statement about the lack of effect on $T_b$ open to questions.

It is good news for drug development that the hyper- and hypothermic side effects of TRPV1 antagonists occur through reverse modulation of the same mechanism. This means that they can be tamed simultaneously, by minimizing the potency of an antagonist to modulate TRPV1 activation by protons. On the structural side, mutational and other studies produced abundant (albeit contradictory) data[50,58] that suggest that this can be achieved. If an antagonist binds to the capsaicin site close to Thr550,[59] this location is relatively remote from the 2 amino acids that are crucial for the proton-induced activation, Glu600 and Glu648.[60-62] Indeed, TRPV1 activation by protons can be abrogated by single-residue mutations without any effect on the channel activation by capsaicin or heat.[63] One way to create a thermally neutral, modality-selective TRPV1 antagonist would be to aim at binding to the capsaicin site without causing conformational changes that would interfere with the activation by protons, whereas both hyper- and hypothermia-inducing TRPV1 antagonists are likely to allosterically modulate TRPV1 activation by protons by enforcing a conformation that makes the channel either insensitive or hypersensitive to this stimulus.

A thermally neutral TRPV1 antagonist that has been shown to interact with TRPV1 according to the proposed scenario is capsazepine. It binds to the capsaicin-sensitive domain of the rat TRPV1 channel between residues 481 and 550[62] and works as an orthosteric antagonist against capsaicin, while having no allosteric effect on proton activation.[47,64] Accordingly, it is efficacious in rat models of inflammatory pain and heat hyperalgesia,[65-67] but does not affect $T_b$ in rats.[13]

Obtaining in-protein crystal structures of thermally neutral, hyper- and hypothermic antagonists of the same chemotype (eg chromanyl ureas reported by Gomtsyan et al[22]) would be required to propel highly targeted, structure-driven design. Given that modern techniques produce sufficiently accurate TRPV1 structures to resolve side-chain conformations in various environments[68-70] and that the accurate structural information for TRPV1 in complex with various agonists and antagonists is already available (see, eg ref. 68), an explosion of rational design of TRPV1 antagonists is expected. Perhaps it has already started.[45,50,71-73]

## 3.5 | Physiological significance

Potent and selective pharmacological antagonists constitute, arguably, the best tool for revealing tonically active physiological mechanisms.[74] Indeed, an antagonist blocks only processes that already occur in the body. In contrast, a pharmacological agonist may produce effects that never occur under natural conditions, because an endogenous agonist may not exist at all, or it may never reach the relevant target at the concentration required. Using genetic models can be tricky as well, because such models are often ridden with multiple processes of chronic genetic and functional compensation.[74] In the past, using the TRPV1 antagonist AMG0347, we have revealed the "unusual" thermoregulatory reflexes, which adjust the activity of autonomic thermoeffectors in rats (non-shivering thermogenesis and tail-skin vasoconstriction) not to the $T_b$, but to the level of pH in yet unidentified abdominal organs, perhaps viscera or muscles.[6] Contrary to the popular beliefs that TRPV1 antagonists cause hyperthermia by blocking TRPV1-mediated warmth sensing,[25,75] blocking thermal signals does not contribute to the hyperthermia,[6,13] as the mammalian $T_b$ regulation system does not use the TRPV1 channel as a temperature sensor.[17] The hyperthermic response to TRPV1 antagonists occurs due to a blockade of the reflexive, pH-driven inhibition of thermogenesis and cutaneous vasoconstriction.[6]

The present study extends our understanding of the physiology of these unusual reflexes that link pH and $T_b$. The fact that the potentiation of proton-mediated TRPV1 activation by TRPV1 antagonists causes hypothermia suggests that these reflexes operate in a wide range of pH. Not only can they remove the inhibition of cold-defence effectors (when proton activation is blocked, or when pH is high), but they also can enhance this inhibition (when proton activation is potentiated, or when pH is low). Hence, these reflexes present a unique physiological mechanism that, via reverse modulation, can bring about either hyper- or hypothermia. This is highly unusual, as the 2 responses

typically utilize different mechanisms, even when triggered by the same compounds (see Introduction).

The physiological significance of these reflexes is speculative, but because polymodal TRPV1 antagonists induce robust hyperthermia in different species,[5] it is probably related to some basic physiological interactions. Originally, we thought that interactions between the feeding status, gastrointestinal pH and $T_b$ are involved.[17] However, in view of our recent results showing that vagotomy does not affect the hyperthermic response to TRPV1 antagonists (A. Garami, A. A. Steiner, and A. A. Romanovsky, unpublished observations), this scenario can be dismissed. Rather, we now think about interactions between physical activity, acid-base homoeostasis and $T_b$. Strenuous physical activity causes metabolic acidosis, including marked acidaemia,[76] and it increases deep $T_b$ and often peripheral temperatures. Based on the tight co-expression of TRPV1 with acid-sensing ion channel-3 on metaboreceptive afferents in muscle arterioles, it has been proposed that TRPV1 channels at this location may function as sensors for reflexes triggered by the acidic environment and elevated temperature of working muscles,[77] although the authors were thinking about axon reflexes. When physical activity is especially strenuous (eg when an animal is running from a predator for life), body temperatures can reach very high values. For instance, an abdominal temperature of >47°C was recorded in a running gazelle.[78] Yet, high body temperatures, whether peripheral,[79] central[80] or both,[81] are well known to reduce physical performance. Hence, a vicious circle is formed, in which an animal has to run as fast as it can to survive, which greatly increases its body temperatures, which, in turn, decreases its ability to run. In such circumstances, it would be highly beneficial to counteract the development of hyperthermia by eliminating cold-defence responses (thermoregulatory heat conservation and heat production). The reflexes discussed here may do just that. When an animal runs, its internal environment acidifies, and the low pH, via TRPV1, inhibits cold defences, thus bringing $T_b$ down. This speculative line of thought deserves experimental studies.

## 4 | MATERIALS AND METHODS

### 4.1 | Synthesis of A-1165901

We synthesized 1-((*R*)-2,2-diethyl-6-fluoro-chroman-4-yl)-3-(1-methyl-isoquinolon-5-yl)-urea, named A-1165901 (Figure 9), as described below.

#### 4.1.1 | Step 1

1-Methyl-5-nitroisoquinoline (2.19 g, 11.64 mmol)[82] was dissolved in methanol/tetrahydrofuran (20 mL, 1:1) in a 250-mL stainless steel pressure bottle, to which 5% Pd-C (0.438 g, 4.12 mmol) was added, and which was then stirred for 2 hour under 30 psi hydrogen at room temperature.

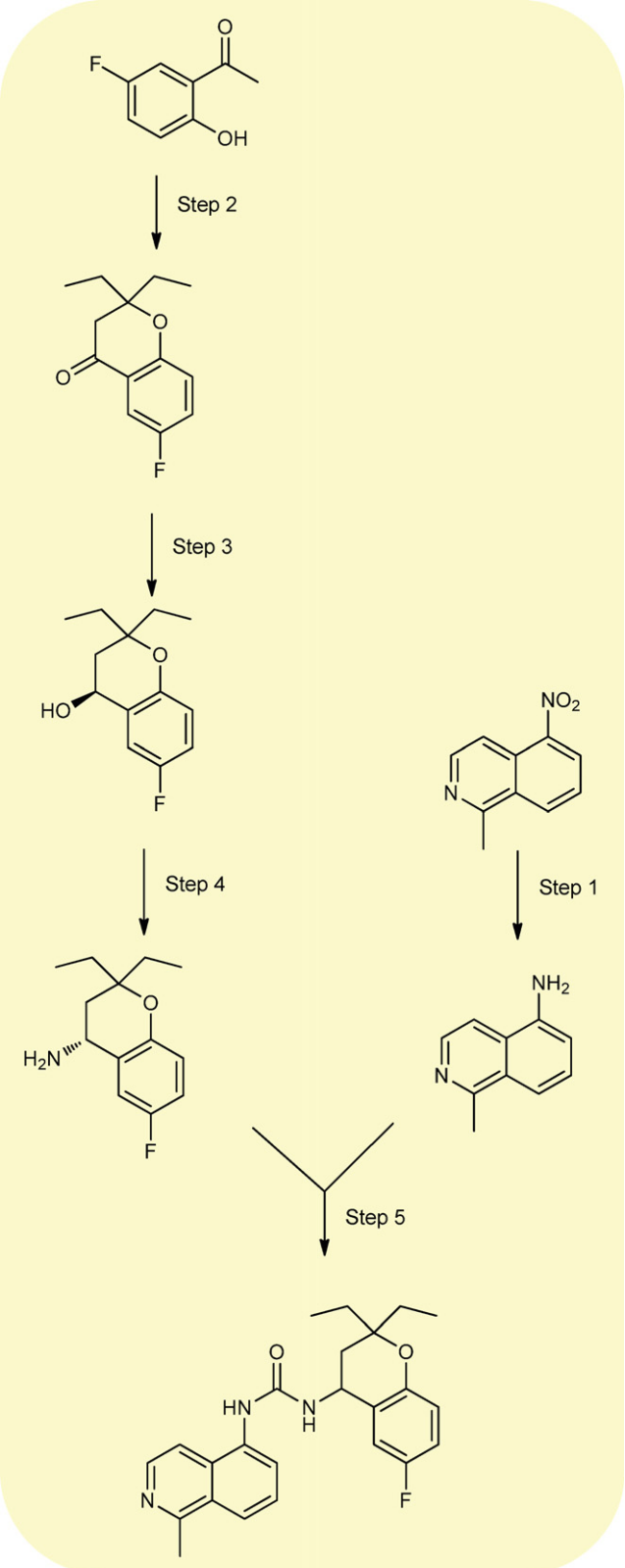


**FIGURE 9** The synthesis of A-1165901 (1-((*R*)-2,2-diethyl-6-fluoro-chroman-4-yl)-3-(1-methyl-isoquinolon-5-yl)-urea)

The mixture was filtered through a nylon membrane and the volatiles evaporated in vacuo. The resulting greyish solid was triturated with 1:1 hexanes:$CH_2Cl_2$ (50 mL) to afford 1-methylisoquinoline-5-amine (1.62 g, 88% yield) as an off-white solid.$^1$H NMR (300 MHz, DMSO) δ 8.28-8.12 (m, 1H), 7.79 (d, $J$ = 6.0 Hz, 1H), 7.39-7.26 (m, 2H), 6.86 (dd, $J$ = 6.7, 1.8 Hz, 1H), 5.90 (bs, 2H), 2.79 (s, 3H). Ammonia direct chemical ionization (DCI/$NH_3$) MS m/z 159 $(M+H)^+$.

### 4.1.2 | Step 2

1-(5-Fluoro-2-hydroxyphenyl)ethanone (30.2 g, 196 mmol) and MeOH (300 mL) were stirred at room temperature, and 3-pentanone (41.6 mL, 392 mmol) and pyrrolidine (17.8 mL, 216 mmol) were added. The mixture was heated to 60°C for 62 hours, at which point LC-MS analysis showed clean conversion to product. The reaction was cooled and concentrated to a minimal volume of MeOH, and methyl *tert*-butyl ether (MTBE, 300 mL) was added. The organics were washed with 2N HCl (150 mL), brine (60 mL), 2N NaOH (150 mL) and brine (60 mL). The solution was passed through a plug of silica gel (30 g), washing with MTBE (150 mL). The filtrate was concentrated, giving 2,2-diethyl-6-fluorochroman-4-one (38.8 g, 175 mmol, 89%) as a light brown oil. $^1$H NMR (300 MHz, DMSO) δ 7.50-7.36 (m, 2H), 7.06 (dd, $J$ = 8.9, 4.3 Hz, 1H), 2.79 (s, 2H), 1.80-1.58 (m, 4H), 0.86 (t, $J$ = 7.5 Hz, 6H). MS (DCI/$NH_3$) m/z 240 $(M+NH_4)^+$.

### 4.1.3 | Step 3

To a solution of (*R*)-(+)-α,α-diphenyl-2-pyrrolidinemethanol (1.322 g, 5.22 mmol) and borane-*N*,*N*-diethylaniline complex (22.27 mL, 125 mmol) in MTBE (100 mL) at 45°C was added a solution of 2,2-diethyl-8-fluorochroman-4-one (23.20 g, 104 mmol) in MTBE (100 mL) slowly (over ~90 minutes). The reaction mixture was stirred for 15 minutes and cooled to 5°C, and MeOH (100 mL) was added, keeping the reaction below 10°C ($H_2$ evolution). After 30 minutes at room temperature, the reaction was diluted with MTBE (100 mL) and washed with 2N HCl (50 mL, 2×) and brine (50 mL), dried ($Na_2SO_4$), filtered and concentrated to obtain 23.4 g of crude material as a yellow oil (ee >98% from chiral HPLC with Chiralcel OJ column eluting with 30% IPA/hexane), which was used without further purification. $^1$H NMR (300 MHz, DMSO) δ 7.15 (ddd, $J$ = 9.4, 3.2, 0.9 Hz, 1H), 6.92 (m, 1H), 6.71 (dd, $J$ = 8.9, 4.8 Hz, 1H), 5.43 (d, $J$ = 6.2 Hz, 1H), 4.63 (m, 1H), 2.14 (m, 1H), 1.75-1.40 (m, 5H), 0.89-0.78 (m, 6H). MS (DCI/$NH_3$) m/z 225 $(M+H)^+$.

### 4.1.4 | Step 4

In a 500-mL flask, (*S*)-2,2-diethyl-6-fluorochroman-4-ol (4.25 g, 18.95 mmol) and *N*,*N*-diisopropylethylamine (16.7 mL, 96.6 mmol) in 60 mL of tetrahydrofuran (THF) were added to give a yellow solution. The reaction mixture was cooled to −40°C, and methanesulfonic anhydride (11.22 g, 64.4 mmol) was added as a solid. After stirring at 30°C for 2 hour, tetra-*N*-butylammonium azide (10.78 g, 37.9 mmol) was added as a solid at −30°C and the reaction mixture was allowed to warm to room temperature overnight. Then, 200 mL of MeOH and 50 mL of 2N NaOH were added and the stirring continued for 30 minutes. The reaction mixture was diluted with MTBE (200 mL) and washed with 2N NaOH (50 mL), water (50 mL), 2N HCl (50 mL, 2×) and water (50 mL), dried ($Na_2SO_4$), filtered and concentrated to obtain crude azide. $^1$H NMR (300 MHz, DMSO) δ 7.38-6.85 (m, 2H), 6.77 (m, 1H), 4.88 (m, 1H), 3.62 (s, 1H), 2.25 (dd, $J$ = 13.8, 6.3 Hz, 1H), 1.85 (dd, $J$ = 13.9, 8.7 Hz, 1H), 1.75-1.44 (m, 4H), 0.93-0.80 (m, 6H).

The crude azide was taken up in THF (200 mL) and water (20 mL). Triphenylphosphine (5.47 g, 20.85 mmol) was added at room temperature, and the reaction mixture was stirred at 50°C for 3 hour and at room temperature overnight. The reaction was concentrated, diluted with dichloromethane (DCM, 200 mL) and washed with 2N HCl (100 mL, 2×). The aqueous phase was basified with 2N NaOH and washed with DCM (200 mL, 6×), dried ($Na_2SO_4$) and concentrated. The residue was purified on $SiO_2$ (0-8% MeOH/$CH_2Cl_2$) to give the final product (2.48 g, 59%), ee 91% from chiral HPLC with Chiralcel OJ column eluting with 21-50% IPA/hexane. MS (DCI/$NH_3$) m/z 224 $(M+H)^+$.

### 4.1.5 | Step 5

Phenyl carbonochloridate (0.239 mL, 1.896 mmol) was added dropwise to a solution of 1-methylisoquinoline-5-amine (0.3 g, 1.896 mmol) and pyridine (0.153 mL, 1.896 mmol) in $CH_2Cl_2$ (20 mL) at 0°C and stirred for 15 minutes. Hunig's base (0.828 mL, 4.74 mmol) was added, followed by the addition of (*R*)-2,2-diethyl-6-fluorochroman-4-amine (0.590 g, 1.580 mmol) in 1 portion. The cooling bath was removed, and the solution was warmed to room temperature with stirring for 2.5 hour. The solution was then quenched with 1N HCl and diluted with water. The organic phase was washed with brine, dried over $Na_2SO_4$ and filtered and the volatiles evaporated in vacuo. The resulting residue was chromatographed on an Analogix $SiO_2$ column (SuperFlash-25, 40 g) eluted with 5-10% methanol/methylene chloride to afford the desired product (0.55 g, 85% yield) as a white solid. $^1$H NMR (300 MHz,

DMSO) δ 8.68 (s, 1H), 8.39 (d, $J$ = 6.1, 1H), 8.31 (dd, $J$ = 0.8, 7.7, 1H), 7.86 (d, $J$ = 8.4, 1H), 7.80 (d, $J$ = 6.1, 1H), 7.65 (t, $J$ = 6.1, 1H), 7.11 (dd, $J$ = 2.6, 9.5, 1H), 7.01 (m, 2H), 6.80 (dd, $J$ = 4.9, 9.0, 1H), 4.97 (m, 1H), 2.89 (s, 3H), 2.20 (dd, $J$ = 6.2, 13.5, 1H), 1.78-1.50 (m, 5H), 0.90 (m, 6H). MS (ESI) m/e 408 $(M+H)^+$. $[\alpha]^{23}{}_D$ +40° (c = 5, MeOH).

## 4.2 | Intracellular $Ca^{2+}$ assays

### 4.2.1 | Total intracellular $Ca^{2+}$ assay

Experiments were performed using a FLIPR-based high-throughput cellular screening system, as described previously.[18] Briefly, effects of a TRPV1 antagonist on the total intracellular $Ca^{2+}$ concentration were evaluated at human or rat TRPV1 channels expressed on recombinant HEK293 cells following a 3-minutes activation with either of 3 agonists, viz., 50 nmol $L^{-1}$ capsaicin, 3 μmol $L^{-1}$ NADA or a pH 5.0 solution of DPBS/MES titrated with 1N HCl. $IC_{50}$ values were calculated from concentration-response curves for capsaicin. Some effects on TRPV1-dependent $Ca^{2+}$ influx can be missed in these experiments, because the signal detected by a fluorometric reader can originate from the intracellular $Ca^{2+}$ release in addition to the uptake of extracellular $Ca^{2+}$. Any significant intracellular $Ca^{2+}$ release in this assay can readily mask changes of the $Ca^{2+}$ influx.

### 4.2.2 | $Ca^{2+}$ influx assay

$^{45}Ca^{2+}$ uptake assay with a scintillation counter measures only the extracellular $Ca^{2+}$ uptake of the cells and therefore serves as a gold standard for revealing pharmacological profiles of compounds against $Ca^{2+}$ channels (such as TRPV1). Stable CHO cell lines stably expressing rat TRPV1 channels were generated as described in detail elsewhere.[59] Two days before the assays were conducted, these cells were seeded in 96-well plates (20,000 cells per well). For assessment of the ability of A-1165901 and AMG7905 to block TRPV1 channel activation by capsaicin, the cells were incubated for 2 minutes at room temperature with capsaicin (500 nmol $L^{-1}$) and the antagonists (200 pmol $L^{-1}$ to 4 μmol $L^{-1}$) in HBSS, pH 7.4, supplemented with BSA (100 μg $mL^{-1}$) and HEPES (1 mmol $L^{-1}$). $^{45}Ca^{2+}$ (MP Biomedicals, Irvine, CA, USA) in Ham's F-12 medium was then added to achieve a final concentration of 10 μCi $mL^{-1}$, and the cells were incubated for an additional 2 minutes at room temperature. At the end of the second incubation, the wells containing the cells were thoroughly washed with PBS (10 μmol $L^{-1}$; pH 7.4) containing BSA (100 μg $mL^{-1}$). The amount of $^{45}Ca^{2+}$ in the cells was measured using a scintillation counter (MicroBeta Jet; PerkinElmer Life and Analytical Sciences, Boston, MA, USA). For assessment of the effects of A-1165901 and AMG7905 on TRPV1 channel activation by protons, the cells were initially incubated for 2 minutes at room temperature with either of the antagonists in an acid buffer (MES buffer, pH 5.0) supplemented with HEPES (30 mmol $L^{-1}$), after which the assay proceeded as described above for the activation by capsaicin assay.

## 4.3 | Animals

Physiological experiments were performed in mice and rats at St. Joseph's Hospital and Medical Center, University of Pecs, AbbVie and Amgen, under protocols approved by their respective Institutional Animal Use and Care Committee. *Trpv1*$^{-/-}$ and *Trpv1*$^{+/+}$ mice were obtained from the Laboratory Animal Centre of the University of Pecs, where they were bred from breeding pairs generously donated by J. B. Davis.[83] Forty-six mice of both sexes were used in the experiments performed at the University of Pecs. At the time of the experiments, the mice weighed 18-27 g. Additional 12 *Trpv1*$^{-/-}$ and 10 *Trpv1*$^{+/+}$ adult male mice (20-30 g) were obtained from the Amgen colony at Charles River Laboratories and were used in the experiments at Amgen. Fourteen male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA), weighing 200-300 g, were housed and used in the experiments at AbbVie. Forty male Wistar rats (Harlan, Indianapolis, IN, USA), weighing 290-380 g, were housed and used in the experiments at St. Joseph's Hospital and Medical Center. At all 4 centres, animals were housed in temperature-controlled rooms on a 12/12-hour light/dark cycle. Standard rodent chow and tap water were available ad libitum. To minimize the stress resulting from drug administration, at the University of Pecs mice were handled and habituated to staying inside wire-mesh cylindrical confiners (3 × 15 minutes daily for 8 days). For the thermophysiological experiments at St. Joseph's Hospital and Medical Center, rats were extensively habituated to experimental conditions in the thermocouple and respirometry set-ups (where they stayed in cylindrical confiners), as described elsewhere.[84]

## 4.4 | Surgeries

Each mouse or rat was subjected to one of the surgical procedures described below. Experiments were performed 3-6 days after the surgery, except for the telemetry experiments in rats, where the animals were allowed a 2-weeks post-surgical recovery period before an experiment.

### 4.4.1 | Mice

Each mouse assigned to an experiment in the telemetry set-up at Amgen was anaesthetized using 4% isoflurane

(Abbott Laboratories) in oxygen at a gas flow of 4 L $min^{-1}$ and implanted with a radiotelemetry temperature probe (model ER-4000 PDT; Mini-Mitter, Bend, OR, USA) into the peritoneal cavity, as described elsewhere.[85] Each mouse assigned to an experiment in the telemetry set-up at the University of Pecs was anaesthetized with i.p. ketamine-xylazine cocktail (81.7 and 9.3 mg $kg^{-1}$ respectively) and implanted with a miniature telemetry transmitter (G2 E-Mitter series; Mini-Mitter) and a polyethylene (PE)-50 catheter (filled with pyrogen-free saline) into the peritoneal cavity, as described previously.[14,86]

#### 4.4.2 | Rats

Each rat assigned to an experiment in the telemetry set-up was anaesthetized with sevoflurane (Abbott Laboratories) and placed on a heating pad. Following midline laparotomy, a telemetry transmitter (model TA-F40; Data Sciences International, St. Paul, MN, USA) was inserted into the peritoneal cavity and sutured to the abdominal wall. The surgical wound was closed in layers.

A rat designated for an experiment in the thermocouple or respirometry set-up was anaesthetized with ketamine-xylazine-acepromazine (55.6, 5.5 and 1.1 mg $kg^{-1}$ respectively, i.p.) and received enrofloxacin (1.1 mg $kg^{-1}$, s.c.). After a small midline incision, a PE-50 catheter was inserted in the abdominal cavity. Its internal end was fixed to the abdominal wall, and the free end was tunnelled under the skin to the nape, exteriorized and sealed. The surgical wound was sutured in layers. The catheter was flushed on the day after the surgery and every other day thereafter.

### 4.5 | Experimental set-ups

Three experimental set-ups were used: the telemetric thermometry ("telemetry") set-up, the thermocouple thermometry ("thermocouple") set-up and the thermocouple thermometry and respirometry ("respirometry") set-up.

#### 4.5.1 | Telemetry set-up

This set-up was used to measure the effects of TRPV1 antagonists on the deep (abdominal) $T_b$ in freely moving rats and *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ mice. Rats and mice implanted with temperature-measuring devices were housed in a temperature-controlled room (20-22°C). Animals, in their home cages, were placed on telemetry receivers (Data Sciences International for rats or Mini-Mitter for mice). Each receiver was connected to a computer. On the day of an experiment, each animal was placed in a cage with clean bedding and had no access to food or water for the duration of the experiment.

#### 4.5.2 | Thermocouple set-up

This set-up permitted simultaneous recording of deep (colonic) $T_b$ and tail-skin temperature (an indicator of vasomotor tone) in restrained rats that were either desensitized with RTX (see Intra-abdominal desensitization of TRPV1 channels) or sham-desensitized. The thermocouple set-up used has been described in detail previously.[6,36,86] The $T_a$ was set to 27°C, which is slightly subneutral for rats in this set-up.[36,84]

#### 4.5.3 | Respirometry set-up

In this set-up, $\dot{V}O_2$ (an indicator of thermogenesis), as well as deep $T_b$ and tail-skin temperature, was measured in restrained rats, as in the past.[6,36,86] Experiments were performed at a $T_a$ of either 26 or 17°C, which is thermoneutral or subneutral respectively, for rats in this set-up.[6,36]

### 4.6 | Drugs and drug administration

#### 4.6.1 | Intragastric AMG7905 to mice

On the day of the experiment, AMG7905 (Amgen) was dissolved in Tween-80-PEG 400 (1:9) at a concentration of 6 mg $mL^{-1}$. AMG7905 (30 mg $kg^{-1}$) or vehicle was administered by gastric gavage (5 mL $kg^{-1}$).

#### 4.6.2 | Intragastric A-1165901 to rats

On the day of the experiment, A-1665901 (AbbVie) was dissolved in ethanol-Tween-80-PEG 400 (1:2:7) at a concentration of 8.2 mg $mL^{-1}$. A-1165901 (41 mg $kg^{-1}$) or vehicle was administered by gastric gavage (5 mL $kg^{-1}$).

#### 4.6.3 | Intraperitoneal A-1165901 and AMG7905 to mice

On the day of the experiment, A-1165901 or AMG7905 was freshly dissolved in saline containing 5% Tween-80 to make a working solution of 0.75 mg $mL^{-1}$. A-1165901 (10 mg $kg^{-1}$), AMG7905 (10 mg $kg^{-1}$) or vehicle was administered in bolus (~13 mL $kg^{-1}$) via the pre-implanted i.p. catheter. To avoid cooling the mouse, the solution was warmed to 37°C in a water bath immediately before the administration. For the drug administration, the mouse was briefly restrained in a confiner.

#### 4.6.4 | Intraperitoneal A-1165901 to rats

A solution of A-1165901 (0.75 mg $mL^{-1}$) in 5% Tween-80 was prepared and stored at −80°C. On the day of the experiment, the stock solution was thawed and diluted with

5% Tween-80 in saline to make a 0.225 mg $mL^{-1}$ working solution. A-1165901 (3 mg $kg^{-1}$) or vehicle was infused (1.67 mL $kg^{-1}$ $min^{-1}$, 10 minutes) to rats through an extension of the pre-implanted i.p. catheter.

### 4.7 | Intra-abdominal desensitization of TRPV1 channels

To cause localized intra-abdominal desensitization of TRPV1 channels, rats were injected with RTX (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 μg $kg^{-1}$, i.p. A working solution of RTX (20 μg $mL^{-1}$) in 20% ethanol in saline was prepared on the day of the experiment. Because RTX causes discomfort and pain at desensitizing doses, the i.p. injection was performed under ketamine-xylazine-acepromazine (55.6, 5.5 and 1.1 mg $kg^{-1}$ respectively, i.p.) anaesthesia, as in earlier studies.[6,49]

### 4.8 | TRPV1 desensitization tests

In an earlier study,[6] we developed a battery of tests to assess the efficacy and extent of TRPV1 desensitization. Two of these were used in the present work: the writhing test (to assess the function of peritoneal TRPV1 channels) and the eye-wiping test (to assess the function of corneal TRPV1 channels). All tests were performed 6-16 days after the initial administration of RTX (or vehicle).

#### 4.8.1 | Writhing test

A rat was injected with RTX (0.1 μg $kg^{-1}$, i.p.) dissolved in 10% ethanol, and writhing episodes (abdominal muscle contraction associated with hindlimb extension) were counted for 10 minutes.

#### 4.8.2 | Eye-wiping test

A drop (20 μL) of RTX (2 μg $mL^{-1}$) in 10% ethanol was applied to the cornea, and eye-wiping movements were counted for 5 minutes.

### 4.9 | Experimental design, data processing and statistical analysis

The *HLI* and the rate of $VO_2$ were calculated as in the earlier studies.[6,36,86] Data on deep $T_b$ (abdominal or colonic), *HLI* and $VO_2$ were compared by 2-way ANOVA, followed by Fisher's LSD post hoc test, as appropriate. Numbers of wipes and writhes were compared between RTX- and sham-desensitized rats by paired 2-tailed Student's *t* test. Statistica AX'99 (StatSoft, Tulsa, OK, USA) software was used for statistical analyses. Differences were considered significant at $P < .05$. Data are reported in the mean ± SE format.

## CONFLICT OF INTEREST

H.A.M., R.M.R., A.Gom. and P.R.K. are employed by AbbVie, D.X.D.Z., S.G.L. and N.R.G. are employed by Amgen, Inc. A.A.R. is a cofounder of Catalina Pharma, Inc.; he has consulted for 6 thermo-TRP programs at pharmaceutical companies; and his thermo-TRP-related research has been supported by Amgen, Inc., Abbott Laboratories and AbbVie, Inc.

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