A Human TRPA1-Specific Pain Model

Stefan Heber,¹ Markus Gold-Binder,¹ Cosmin I. Ciotu,¹ Martin Witek,² Nino Ninidze,³ Hans-Georg Kress,³ and Michael J.M. Fischer¹

Institutes of ¹Physiology, ²Pharmacology, Center for Physiology and Pharmacology, and ³Division of Special Anesthesia and Pain Medicine, Department of Anesthesia, Intensive Care and Pain Medicine, Medical University of Vienna, 1090 Vienna, Austria

The cation channel transient receptor potential ankyrin 1 (TRPA1) plays an important role in sensing potentially hazardous substances. However, TRPA1 species differences are substantial and limit translational research. TRPA1 agonists tested previously in humans also have other targets. Therefore, the sensation generated by isolated TRPA1 activation in humans is unknown. The availability of 2-chloro-*N*-(4-(4-methoxyphenyl)thiazol-2-yl)-*N*-(3-methoxypropyl)-acetamide (JT010), a potent and specific TRPA1 agonist, allowed us to explore this issue. To corroborate the specificity of JT010, it was investigated whether the TRPA1 antagonist (1E,3E)-1-(4-fluorophenyl)-2-methyl-1-penten-3-one oxime (A-967079) abolishes JT010-elicited pain. Sixteen healthy volunteers of both sexes rated pain due to intraepidermal injections of different concentrations and combinations of the substances. The study design was a double-blind crossover study. All subjects received all types of injections, including a placebo without substances. Injections of the TRPA1 agonist dose-dependently caused pain with a half-maximal effective concentration of 0.31 μ M. Coinjection of A-967079 dose-dependently reduced and at a high concentration abolished JT010-induced pain. Quantification of JT010 by HPLC showed that a substantial part is adsorbed when in contact with polypropylene surfaces, but that this was overcome by handling in glass vials and injection using glass syringes. Isolated TRPA1 activation in humans causes pain. Thus, intradermal JT010 injection can serve as a tool to validate new TRPA1 antagonists concerning target engagement. More importantly, TRPA1-specific tools allow quantification of the TRPA1-dependent component in physiology and pathophysiology.

Key words: A-967079; JT010; pain model; psychophysics; target validation; TRPA1

Significance Statement

This study showed that activation of the ion channel transient receptor potential ankyrin 1 (TRPA1) alone indeed suffices to elicit pain in humans, independent of other receptors previously found to be involved in pain generation. The newly established TRPA1-specific pain model allows different applications. First, it can be tested whether diseases are associated with compromised or exaggerated TRPA1-dependent painful sensations in the skin. Second, it can be investigated whether a new, possibly systemically applied drug directed against TRPA1 engages its target in humans. Further, the general possibility of quantitative inhibition of TRPA1 allows identification of the TRPA1-dependent disease component, given that the substance reaches its target. This contributes to a better understanding of pathophysiology, can lay the basis for new therapeutic approaches, and can bridge the gap between preclinical research and clinical trials.

Introduction

The cation channel transient receptor potential ankyrin 1 (TRPA1) plays an important role in pain perception and is,

among other potential applications, a promising target investigated for novel analgesics (Chen and Hackos, 2015). It is expressed in a substantial fraction of peripheral neurons (Nagata et al., 2005; Bautista et al., 2013; Usoskin et al., 2015; Li et al., 2016; da Silva Serra et al., 2016). TRPA1 acts as a sensor for irritating exogenous (Bandell et al., 2004; Bautista et al., 2006) as well as endogenous substances occurring during oxidative stress and inflammation (Andersson et al., 2008). For example, we found that the ion channel is activated by nitroxyl (Eberhardt et al., 2014) and methylglyoxal, which accumulates under diabetic conditions (Eberhardt et al., 2012). Furthermore, TRPA1 is also activated by a low pH (de la Roche et al., 2013), although this did not translate into a contribution of TRPA1 to the sensation of tissue acidosis in humans (Schwarz et al., 2017).

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Correspondence should be addressed to Stefan Heber at stefan.heber@meduniwien.ac.at.

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In animal studies, TRPA1 acts as a pivotal sensor for potential tissue damage and thereby contributes to the perception of pain, representing a vital function of the nervous system (Obata et al., 2005). This includes inflammatory pain (e.g., in inflamed joints; Moilanen et al., 2015; Horváth et al., 2016) or visceral inflammation like colitis and pancreatitis (Mitrovic et al., 2010; Schwartz et al., 2013), but also neuropathic pain (Naik et al., 2006; Trevisan et al., 2016). Further, it plays a role in secondary inflammation after ischemia (Hamilton et al., 2016) and chemotherapy-induced peripheral neuropathic pain (Materazzi et al., 2012; Trevisan et al., 2013) or diabetic neuropathies (Koivisto et al., 2012).

However, a major drawback for pharmaceutical development are the substantial species-dependent differences in TRPA1 function. Major differences were found in response to pharmacological compounds, first described for menthol and in particular concerning noncovalent agonists (Xiao et al., 2008). Considerable differences have also been found between humans and distant species considering thermal stimuli (Cordero-Morales et al., 2011). Such differences were not found for example, for TRP cation channel subfamily V member 1 (TRPV1), and this could impede the TRPA1 drug discovery (Bianchi et al., 2012). Consequently, there is a need for human pain models on TRPA1 that help to bridge the translational gap between preclinical and clinical research.

Previously described chemical stimulation of TRPA1 in human subjects has used substances which are known to coactivate other targets. For example, allyl isothiocyanate (AITC) was recently used for a TRPA1-dependent pain model (Andersen et al., 2017); however, AITC can also activate TRPV1 (Gees et al., 2013). According to the reported EC_{50} of 0.65 nM, 2-chloro-*N*-(4-(4-methoxyphenyl)thiazol-2-yl)-*N*-(3-methoxypropyl)-acetamide (JT010) was the most potent commercially available agonist (Takaya et al., 2015). Further, it was shown to be specific regarding other pain-related TRP channels up to a concentration of 1 μ M. By means of this substance, and the most potent commercially available antagonist (1*E*,3*E*)-1-(4-fluorophenyl)-2-methyl-1-penten-3-one oxime (A-967079), we established a human TRPA1-specific pain model. Here we show that an isolated TRPA1 activation caused pain in humans. This pain could be abolished by a TRPA1 antagonist. The established pain model allows validation of new pharmacological antagonists and elucidation of the role of TRPA1 channels in human disease.

Materials and Methods

Experimental design

In a training session, conducted at least 1 d before the main trial, a subject was familiarized with the rating of pain by separate epidermal injection of control [50 μ l phosphate-buffered synthetic interstitial fluid (PB-SIF)] and capsaicin 3.1 μ M (0.001%) as a positive control. This also allowed the subjects to decide on their further participation in the study.

The main trial had a double-blinded, single-group, randomized, placebo-controlled crossover design. Each of the 16 subjects (representing a single group) sequentially received nine injections that differed only in the concentrations of JT010 and A-967079 (Fig. 1). Both the experimenter and the volunteers were blinded regarding the injected substances. The sequences of the injected substances were predefined by a Latin-squares Williams design, which balances for first-order carryover effects (Williams, 1949). Sequences were generated in R by the “williams”

Figure 1. Study design. About every 5 min, each subject received an intradermal injection of 50 μ l with the indicated concentrations of TRPA1 agonist JT010 and TRPA1 antagonist A-967079. Horizontal gray arrows visualize the period during which pain was typically experienced by the subjects. $\emptyset$ indicates absence of the respective substance.

program of the “crossdes” package. Volunteers were randomly allocated to these sequences.

Participants. Human volunteers of both sexes with full legal capacity between the ages of 19 and 41 years were recruited (inclusion age range, 18–70 years). Exclusion criteria were participation in another study within the last 4 weeks, medication intake (except contraception) or drug abuse, pregnancy or current breastfeeding, fever, allergic diseases, in particular asthmatic disorders and allergic skin diseases, any sensory deficit at the test site (volar forearm), medical history of angina pectoris, thrombosis, tumors, pneumonia, tuberculosis, bleeding diathesis, and diseases that could put the experimenter at risk (e.g., HIV, hepatitis).

Human psychophysical experiments. Experiments with human volunteers were approved by the ethics committee of the Medical University of Vienna (1799/2017) and were performed in accordance with the Declaration of Helsinki after written informed consent. Eight male and eight female subjects completed the study.

For experimental sessions, subjects were seated comfortably, and 50 μ l of each of the above-mentioned solutions were injected into the epidermal layer of the volar forearm using a glass syringe (100 μ l; Hamilton) with an attached cannula (0.30 $\times$ 12 mm; Sterican, Braun). The nine glass syringes were autoclaved after every use. Test sites were at least 5 cm apart and did not match the capsaicin injection site of the training session. There was a minimum period of 5 min between injections, which resulted in a duration of $\sim$ 45 min for the main trial. The right and left arm were used in alternating sequence for the injections. Pain was rated by means of a numeric rating scale (range, 0–100 0 for “no pain”; 100 for “maximum imaginable pain”). After insertion of the thin cannula, volunteers rated the resulting pain of this insertion by a single number (insertion pain). The solution was injected once the insertion pain had fully subsided. After each injection, subjects provided a pain rating every 5 s until six subsequent zero ratings were acquired. The area under the curve of pain (pain AUC) ratings was calculated as the sum of all pain ratings. This integral measure of pain after each injection was taken as the primary outcome variable. The primary hypothesis that the injection of 1 μ M JT010 causes pain was tested by comparing the pain AUC values between the control injection (PB-SIF) and the injection containing 1 μ M JT010 (without A-967079). Second, we hypothesized that simultaneous coinjection of the TRPA1 antagonist A-967079 reduces pain induced by JT010. For this purpose, the injection containing 1 μ M JT010 and the one containing 1 μ M JT010 together with 10 μ M A-967079 were tested against each other.

Randomization, sequence generation, and blinding. The three pivotal injections for testing the two main hypotheses were placed at injection numbers 2, 4, and 6 in a balanced design to achieve a limited and defined time between these three injections as well as to avoid first-order carryover effects. The remaining six positions were also filled by Latin squares-based sequences with a balanced design. Sequences were stored in identical closed envelopes. One envelope was randomly picked and discarded to define the sequence of the injected substances for each volunteer. To conceal the identity of prepared solutions both from the experimenter and the subjects, a third person prepared the solutions immediately before their experimental use and handed them to the blinded experimenter.

Statistical analysis

Based on a pilot experiment, which was also used to find an experimentally suitable JT010 concentration, we expected a pain AUC induced by

JT010 1 μM of ~ 400 units. We assumed that the pain AUC in response to JT010 1 μM + A-967079 10 μM can only be in the range between the pain AUC in response to the control injection and the JT010 1 μM injection. Thus, the effect size concerning the secondary hypothesis was necessarily less or equal compared with the primary hypothesis, requiring an equal or greater sample size. Therefore, sample size estimation was based on the secondary hypothesis. We considered a pain AUC reduction by 10 μM A-967079 of 50% as relevant, corresponding to 200 units. Based on previous studies (Schwarz et al., 2017), we expected the SD of the differences of two injections in the same individual to be ~ 220 units. To detect a 50% reduction in pain AUC with a power of 0.8, accepting a type I error probability of 0.05 and the assumed SD of differences, 12 volunteers were needed. We expected that up to 25% of all volunteers, who initially agreed to take part in this trial, could drop out after the session for familiarization. Therefore, we included a total of 16 volunteers, who all completed the main trial.

The primary statistical analysis was performed by a mixed linear model with pain AUC as a dependent variable and an identity covariance structure. The model included the subjects as levels of a random factor and “injection type” as fixed within-subject factor with nine levels according to the nine injections. To account for the crossover situation, the order of injections was included in the model as a second-degree polynomial. Planned contrasts were used to test predefined hypotheses: the primary hypothesis was tested by comparing the pain AUC values between the control injection (PB-SIF) and the injection containing 1 μM JT010 (without A-967079). In case the primary null hypothesis had been rejected, the secondary hypothesis was tested by comparing the injections containing 1 μM JT010 without A-967079 and the one containing 1 μM JT010 plus 10 μM A-967079. Additional contrasts were performed in an exploratory manner without adjustment for multiple testing. Due to different variances between groups (heteroscedasticity), the main statistical analysis was performed with square-root-transformed pain AUC values. Unless stated otherwise, graphs show back-transformed least-squares means (adjusted for the order of injections) with SEs on the original scale. To explore the effect of insertion pain on the pain AUC, it was added as a metric predictor to the primary mixed-linear model.

EC_{50} and IC_{50} values were estimated by fitting a four-parameter logistic dose–response curve to estimated means resulting from the mixed linear model. For combined presentation, *in vivo* and *in vitro* data were normalized by setting the maximum response to 100%. The percentage of inhibition achieved by A-967079 10 μM was calculated for each individual separately using the following formula:

$$\frac{\text{AUC Pain}_{\text{JT010 } 1\mu\text{M}} - \text{AUC Pain}_{\text{A-967079 } 10\mu\text{M}}}{\text{AUC Pain}_{\text{JT010 } 1\mu\text{M}} - \text{AUC Pain}_{\text{PB-SIF}}} \times 100.$$

Thereafter the median percentage with its 95% confidence interval (CI) was determined by bias-corrected and accelerated bootstrapping with 1000 samples. The maximum pain rating and seconds until maximum pain, half-maximal pain, and time until pain has subsided were compared using a linear model with the three injections as within-subjects factor. Statistical analyses were performed using IBM SPSS Statistics 24 and GraphPad Prism 7. Two-sided p values ≤ 0.05 were considered statistically significant. Results are reported as the mean $\pm$ SEM, for datasets with skewed distribution median and 95% confidence intervals are reported as indicated.

Chemicals and solutions

As in the previous study (Schwarz et al., 2017), a PB-SIF was used, in which all substances were dissolved. This is a modification of the synthetic interstitial fluid by Bretag (1969) in which hydrogen carbonate was replaced by dihydrogen phosphate, as described previously (Schwarz et al., 2017). The PB-SIF contains the following (in mM): sodium chloride 108, potassium chloride 3.5, disodium hydrogen phosphate 23, magnesium sulfate 0.7, calcium chloride 1.5, sodium gluconate 9.6, glucose 5.5, and sucrose 7.6. The resulting pH 7.4 was controlled and titrated with sodium hydroxide or hydrogen chloride if necessary. Sterile solutions were obtained by using a 0.22 μm syringe filter. PB-SIF was used as a control solution for all experiments and for diluting sterile stock solu-

tions. JT010, A-967079, N-vanillylnonanamide, GSK1016790A, and pregnenolone sulfate were purchased from Sigma-Aldrich. Capsaicin was dissolved in ethanol (10 mM) and diluted to 3.1 μM with PB-SIF before injection. Acetonitrile [ACN; high-performance liquid chromatography (HPLC) LC-MS grade] was obtained from VWR, formic acid >98% was obtained from Applichem, methanol >99% from Chem-Lab, and ultra-pure water was obtained from our Milli-Q plus system (Millipore).

Special handling of JT010

The volunteer study had to be performed twice due the special properties of JT010. Handling of the substance was adapted for the second round.

Psychophysics experiments round 1. All pipetting steps were performed in 1.5 ml polypropylene tubes (Safe-Lock Tubes, Eppendorf) and with polypropylene pipette tips. A 10 mM stock solution was generated in DMSO, while further dilutions were then prepared in PB-SIF. Injections were performed with 0.3 ml polypropylene syringes with a 27 gauge cannula (BD).

Psychophysics experiments round 2. The stock solution in DMSO was handled with polypropylene tips, while diluted solutions were maintained in glass vials and handled by means of Hamilton syringes. For both JT010 and A-967079, 10 mM stock solutions in DMSO were generated. From these, nine solutions containing 1000-fold the target concentrations of both substances were prepared in DMSO. Aliquots of 1.5 μl were stored in glass vials at -20°C until use. Immediately before application into the epidermis of human subjects, 1498.5 μl PB-SIF was added and the solution vortexed, leading to a final DMSO concentration of 0.1% in all injection solutions (including for the control solution without JT010). These solutions were not exposed to polypropylene tips or tubes during processing to avoid adsorption.

Solid-phase extraction and chromatography analysis

To investigate the loss of JT010 after contact with polypropylene surfaces, the substance was quantified by HPLC. JT010 was dissolved in deionized water and quantified using RP-C18 columns (150 $\times$ 3.0 mm, 2.5 μm ; Kinetex, Phenomenex), in a Dionex Ultimate 3000 UHPLC focused System. The mobile phase composed of solvent A (100% water containing 0.05% trifluoroacetic acid) and solvent B [water/acetonitrile (10:90, v/v) containing 0.05% trifluoroacetic acid] was used with a flow rate of 0.3 ml/min. The gradient used started at 50% solvent B and was linearly raised to 100% solvent B within 10 min, which was continued for a further 5 min. Afterward, the column was re-equilibrated to 50% solvent A in 5 min. Absorption was monitored for 215, 254, 280, and 320 nm.

HPLC with subsequent mass spectroscopy

An ACQUITY Arc chromatographic system was used, consisting of a Sample Manager FTN-R, a Quaternary Solvent Manager-R, a column oven, an ACQUITY QDa, a Single Quadrupole Mass Spectrometer, and MassLynx V4.1 software (Waters). The mobile phase consisted of 50% water, 50% acetonitrile, and 0.1% formic acid, and was mixed by the solvent manager at isocratic conditions with a flow rate of 0.5 ml/min. JT010 2 μM was dissolved in the mobile phase. The Luna C18 2.5 μm HST 50 $\times$ 2 mm (Phenomenex) column was heated to 40°C ; the autosampler was adjusted to 25°C . Data were acquired in single ion record mode, and total ion count in scan mode. Ionization was performed with electrospray ionization (ESI) in positive and negative mode. For both single ion record and total ion count, the capillary voltage was 0.8 kV and the cone voltage was 15 V. The probe temperature was 600°C , and the scan range was from 200 to 1000 m/z (mass/charge ratio). For single ion record the m/z was adjusted to 355 in ESI-positive mode for JT010. Mass distribution over the whole peak chromatographic output was evaluated. All measurements were performed at least fivefold.

Intracellular calcium measurements

CHO cells stably expressing human TRPA1 were provided by Stuart Bevan (King's College London, London, UK). HEK293t cells were transfected with plasmids using jetPEI transfection reagent (Polyplus). The human TRPA1 plasmid was obtained from Ardem Patapoutian (Scripps Research, La Jolla, CA), hTRPA1 was mutated and subcloned to human TRPA1-C621S-C641S-C665S (Meents et al., 2016), human TRPV1 was

Figure 2. JT010 injection causes pain in human subjects. **A**, JT010 dissolved in 50 μl PB-SIF was injected intradermally into the volar forearm. Pain ratings are visualized with median and 95% confidence interval. **B**, JT010 induced a concentration-dependent increase in the pain AUC. **C**, Pain ratings increased rapidly and reached a maximum (100%) 25 s after injection, halved (50%) after 66 s, and disappeared (0%) after 108 s (mean values). **D**, Median pain ratings with 95% confidence intervals after capsaicin injections. *** $p < 0.001$.

obtained from Carla Nau (Universitätsklinikum Schleswig-Holstein, Lübeck, Germany; Leffler et al., 2008), human TRPV4 was obtained from Xuming Zhang (Aston University, Birmingham, UK), human TRPM3 was obtained from Johannes Oberwinkler (Center for Mind, Brain and Behavior, Marburg, Germany), and human TRPM8 was obtained from Thomas Voets (KU Leuven, Leuven, Belgium). Cells were grown in 96-well black walled plates. Cells were loaded with Calcium 6 for 2 h (Calcium 6 Kit, Molecular Devices) in a physiological solution, containing the following (in mM): 145 NaCl, 5 KCl, 10 glucose, 10 HEPES, 1.25 CaCl_2 , and 1 MgCl_2 , buffered to pH 7.4 with NaOH. According to the manufacturer protocol, cells were not washed as the extracellular dye was chemically quenched. Calcium 6 fluorescence excited at 488 nm every 2.5 s served as an index of intracellular calcium. Assays were performed at 37°C in a FlexStation 3 (Molecular Devices). A volume of 50 μl was pipetted automatically according to a preset protocol into 100 μl of medium in the wells. Fluorescence change relative to baseline fluorescence is reported (dF/F_0).

Results

TRPA1 activation in human skin causes pain

Psychophysical experiments including intradermal JT010 injections showed that pain AUC generally differed among all nine injections (mixed linear model, main effect of factor “injection type”: $F_{(8,118)} = 36.57$, $p < 0.001$). The subsequently estimated contrast corresponding to the main hypothesis showed that an injection containing 1 μM JT010 causes more pain than a control injection (planned contrast: $t_{(118)} = 10.4$, $p < 0.001$; Fig. 2A,B). In addition, we explored the effects of different concentrations, which revealed that both 0.31 and 3.1 μM JT010 significantly caused pain ($p < 0.001$ each, tested against control injection). Notably, a cubic polynomial contrast indicated that the increase of pain due to rising JT010 concentrations significantly levels off at higher JT010 concentrations (cubic trend: $t_{(118)} = -2.7$, $p = 0.008$).

Further characterization of this human pain model showed that maximal pain ratings occur after ~ 30 s, pain ratings halve within ~ 70 s, and pain subsides within 120 s (Fig. 2C). The peak pain after JT010 injection occurs significantly later (median time

25 s) than after the injection of a comparable concentration of capsaicin (median time, 5 s), which was used for familiarization with the pain rating (Wilcoxon signed rank test: $z = 3.46$, $p < 0.001$).

Ratings of pain due to insertion of the cannula were not different between the various solutions (Fig. 3A). To investigate to what extent the sensitivity of each injection spot, reflected by the cannula insertion pain, might have affected the results, insertion pain was added as covariate in the primary statistical model. There was a positive relationship between insertion pain and pain AUC ($t_{(127)} = 2.51$, $p = 0.013$); however, this adjustment did not change the estimated pain ratings to a relevant extent (Fig. 3B).

The quality of pain during the whole set of injections was interrogated by the German version of the Pain Perception Scale (Geissner, 1995); both after the capsaicin injection during the training session and after the main trial. The German version of the Pain Perception Scale was used as all subjects were German native speakers. No relevant differences were found between JT010 and capsaicin (Fig. 4). No volunteer reported a cold sensation.

Discrepancy between the previously reported and observed EC_{50} of JT010

JT010 was the TRPA1 agonist of choice for this study due to its reported specificity and low EC_{50} of 0.65 nM. Surprisingly, the estimated EC_{50} of JT010 based on pain ratings was 308 nM (Fig. 5A). Thus, a cell-based assay was used. In a closely spaced concentration range and 12-fold measurements from 3 separate days, the result was an EC_{50} of 47 nM, which is lower compared with the estimated 308 nM in psychophysical experiments.

A potential temperature dependence and cell line dependence of the EC_{50} was addressed by repetition of the calcium assays in hTRPA1-expressing HEK293t cells. Activating JT010 concentrations induced a 29% higher calcium influx at 25°C compared with 37°C lower temperatures ($F_{(1,91)} = 15.6$, $p < 0.001$, extra sum-of-squares F test), but the EC_{50} was not shifted ($F_{(1,91)} =$

Figure 3. Maximal pain ratings due to insertion of the cannula before injection. **A**, Individual maximal pain ratings due to cannula insertion before injection. Pain ratings elicited by insertion of the cannula did not differ between injection types. **B**, Estimated geometric pain AUC means without (prespecified analysis) and with adjustment for differences in insertion pain. Statistical adjustment of pain AUC values for insertion pain did not alter estimated pain AUC values to a relevant extent, showing that the injection spot did not affect the primary results to a relevant extent.

Figure 4. Pain perception of capsaicin and JT010. The perception was interrogated by the Pain Perception Score. Most volunteers rated affective parameters 1–14 with “1,” corresponding to “not applicable” for both substances (data not shown). The sensory characteristics (parameters, 15–33) of capsaicin and JT010 appear largely identical. It needs to be mentioned that these ratings are from separate sessions and the study was not powered to investigate this exploratory question.

0.07, $p = 0.79$). However, the published EC_{50} of 0.65 nM was based on endpoint reads. Representative calcium traces induced by the application of 100 and 3162 nM JT010 showed that, despite pronounced differences of calcium traces, the endpoints can be quite similar. In contrast, the area under the first 30 s of the curve of calcium signals are a suitable measure to quantify calcium influx and show clear differences between JT010 concentrations (Fig. 5B). Therefore, in this manuscript the area under the curve of calcium signal was used for EC_{50} estimation. An exposure of

the cysteine mutant hTRPA1-C621S-C641S-C665S to JT010 1 μ M and 3.1 μ M shows at best a minimal response compared with the wild-type hTRPA1 ($n = 2$ each; Fig. 5A), confirming a covalent mode of action. As specificity of JT010 was previously only shown up to 1 μ M, we further extended this considering human TRPV1. JT010 1 μ M induced 1.2%, 3.1 μ M 2.8% and 10 μ M 5.6% of the response to capsaicin 2 μ M ($n = 2$ each, data not shown). JT010 1–10 μ M did not activate human TRPV4 and human TRPM3 expressed in HEK293t cells, which could be activated by

reference agonists GSK1016790A and pregnenolone sulfate. Also, JT010 10 μM did not activate TRPM8.

JT010 induced pain was abolished by coapplication of the TRPA1 antagonist A-967079

As expected, coapplied A-967079 10 μM inhibited pain induced by JT010 1 μM (planned contrast corresponding to secondary hypothesis: $t_{(118)} = 8.66$, $p < 0.001$; Fig. 6*A,B*). The results showed a pronounced, concentration-dependent reduction of pain. Pain induced by 1 μM JT010 could be reduced by 98% (median) due to coapplication of 10 μM A-967079. The bootstrapped 95% confidence interval ranges from 96% to 101%; therefore, the results are consistent with complete inhibition (Fig. 6*C*). Exploratory analyses of other concentrations showed that the pain AUC was not significantly reduced by 0.31 and 1 μM A-967079, respectively. Interestingly, maximal pain ratings in response to these injections were markedly lower than in response to 1 μM JT010 alone (Fig. 6*D*), whereas the time course of pain ratings was delayed (Fig. 6*E*). The estimated IC_{50} of A-967079 based on pain AUC was 2.1 μM (Fig. 7).

Results of the first trial attempt

The data presented above represent the second conduction of the trial and were generated with optimized handling of JT010. In a first round, induced pain ratings were substantially lower, and only the injections that were intended to contain JT010 3.1 μM caused more pain than the control injection. Contrary to our expectations, an intended concentration of JT010 1 μM did not cause pain. (Fig. 8*A*).

Subsequently, the reason for such low pain ratings was investigated, and as a result JT010 handling was optimized as described below. This led to a reapproval of a second round by the institutional ethics committee, in which pain ratings were substantially higher. Based on these pain ratings, the actual concentration in solutions with an intended JT010 concentration of 3.1 μM in the first round was estimated to have been only 0.42 μM , corresponding to a substance loss of 86% (Fig. 8*B*).

JT010 contact to polypropylene should be avoided

After conducting the first round of psychophysical experiments, it was reasoned that the substance could have been adsorbed and/or chemically modified by polypropylene tubes and pipette tips. To further address this issue, aqueous JT010 solutions, prepared in glass vials, were put into polypropylene tubes, whereupon the JT010 concentration was again quantified by HPLC. Results showed that the concentration was reduced to $67 \pm 1\%$ after a single polypropylene contact. To differentiate between adsorption and chemical reaction, the lipophilic solvent acetonitrile was used to detach JT010 adhering to polypropylene tubes. Acetonitrile recovered $23 \pm 1\%$ of the initial JT010 amount from the polypropylene tube in chemically unaltered form. In sum, the

Figure 5. JT010 concentration response *in vitro* and *in vivo*. **A**, Concentration response of the normalized AUC *in vitro* (solid, measured at 37°C) and *in vivo* (dotted) including half-maximal effective JT010 concentrations (horizontal error bars, 95% CI of EC_{50}). Closed circles show the response to JT010 in HEK293T cells expressing hTRPA1-C621S-C641S-C665S normalized to hTRPA1. **B**, Time course of Calcium 6-reported intracellular calcium level upon application of JT010 at $t = 0$ in a fluorescent plate reader (mean of four replicates). A rapid concentration-dependent increase in the signal rise rate and the peak fluorescence was observed. The respective concentration dependence cannot be detected by an endpoint read (filled circle) and was analyzed by an AUC (gray area) of the first 30 s after JT010 application.

fate of 90% of the initial JT010 amount could be clarified (Fig. 9*A,B*).

Next, the loss of JT010 due to tubes and tip contact was investigated. Repetitive HPLC from the same glass vials as control shows that a JT010 10 μM concentration remains nearly constant over 3 h in glass vials without polypropylene contact. To explore how much substance is lost due to polypropylene tip contact, a second group of JT010 solutions, stored in glass vials, was exposed twice to the same polypropylene pipette between measurements. In a third group, probes were pipetted into polypropylene tubes using polypropylene tips and vortexed, whereupon the solution was transferred after 5 min with the same pipette tip back into the same glass vial, corresponding to two pipette tip contacts and an additional polypropylene tube contact (Fig. 9*C*). Subtraction of the loss observed in group two from the loss observed in group three allowed to estimate the loss by polypropylene tube contact alone. A two-way ANCOVA with baseline values as covariate, time as a within-subjects factor, and group as a between-subjects factor showed that the percentage change of JT010 concentration from the respective previous measurement was not different for each time point ($n = 3/\text{group}$; time main effect, $p = 0.3$; time $\times$ group interaction, $p = 0.5$), but differed among the three groups (group main effect, $p < 0.001$). After every hour in a glass vial, the measured JT010 concentration was $0.8 \pm 0.3\%$ lower. Every double contact to polypropylene tips reduced the

Figure 6. JT010-induced pain is abolished by A-967079 in healthy human subjects. **A**, A-967079 was coinjected with JT010 1 μ M intradermally in 50 μ l into the volar forearm. Pain ratings are presented as the median with 95% confidence interval. **B**, A-967079 inhibited the pain AUC, the highest concentration was not different from a control injection without JT010. **C**, A-967079 10 μ M median inhibition of JT010-induced pain was estimated to be 98% (visualized with 95% CI, which includes 100% inhibition). **D**, **E**, Although the lower concentrations of A-967079 did not reduce the pain AUC, the maximum pain rating was lower (**D**), and the time course of reported pain was more delayed (**E**). This applies to the time of maximum pain (diamond), the time of half-maximal pain (circle), and the time when pain subsided (square). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 7. IC_{50} of A-967079 in the human pain model. Based on the area under the curve of pain ratings after injection, the IC_{50} value and the respective 95% confidence interval were estimated.

amount of remaining JT010 by $11.2 \pm 0.4\%$ (first vs second group, Sidak *post hoc* test, $p < 0.001$), every contact to polypropylene tubes reduced this further by $16.1 \pm 0.4\%$ (second vs third group, Sidak *post hoc* test, $p < 0.001$). Each tube contact including pipetting resulted in a reduction by $27.3 \pm 0.4\%$ (first vs third group, Sidak *post hoc* test, $p < 0.001$).

It was hypothesized that substance loss due to adherence and/or chemical modification is reduced if JT010 is dissolved in a more lipophilic solvent, in particular DMSO. Hence, a defined amount of JT010 was either dissolved in H₂O or DMSO in glass vials ($n = 5$ each). Subsequently, each probe was pipetted with polypropylene tips into polypropylene tubes, out of which the

solutions were removed again with polypropylene tips into glass vials. This procedure was repeated a second time. The elution from the chromatography column showed a peak at 2.5 min, which was absent in blank controls (Fig. 9D). The mass spectrum of this peak showed a mass of 355 Da, which coincides with the published mass of JT010 (Fig. 9E). To quantify JT010 loss in H₂O or DMSO, measurements were repeated by exclusively recording ions with a mass of 355 Da (single ion record). Results showed that the area under the JT010 peak is diminished to a greater extent due to polypropylene contact in H₂O compared with DMSO as the solvent (two-way ANOVA with time as a within-subject factor and solvent as a between-subject factor; time $\times$ solvent interaction, $p < 0.001$; difference between

H₂O and DMSO after one and two tip contacts, $p < 0.001$ each; Fig. 9F, G). The scan mode with positive and negative ionization revealed further peaks. However, these also occurred in solutions with contact to polypropylene only, including JT010-free controls, and can therefore be considered a result of desorption from the “clean” reaction tubes. The results do not support new chemical derivatives arising from JT010, at least not exceeding a marginal level. Based on the observed adsorption, handling of JT010 solutions was adjusted to minimize polypropylene contact for aqueous JT010 solutions. This was achieved by using glass HPLC vials and Hamilton glass syringes.

Figure 8. Results of the first round of psychophysical experiments. **A**, Pain AUC values with conventional substance handling using polypropylene materials were lower than expected. **B**, Based on the concentration–response curve of the second round of experiments with optimized JT010 handling without polypropylene contact, the observed response to an intended concentration of 3.1 μM only equals to what can be expected from 0.42 μM remaining substance. *** $p < 0.001$.

Figure 9. JT010 requires special handling. **A**, To identify sources of substance loss, JT010 10 μM in a glass vial was measured by HPLC (original solution). The substance was poured into a polypropylene reaction tube (1 $\times$ tube contact). JT010 could be recovered by an acetonitrile wash, but a second wash with acetonitrile did not recover further JT010. **B**, Threefold repetition shows 67% of JT010 in solution, and 23% could be desorbed by acetonitrile. **C**, Repetitive HPLC of JT010 10 μM from the same glass vial (diamonds), after contact with the same polypropylene pipette tip twice between measurements (circles), and after transfer into a polypropylene tube and back into the same glass vial by the same polypropylene tip after 5 min (squares). Every contact to polypropylene caused a substantial substance loss. **D**, Representative HPLC protocol that detected the injected JT010 2 μM at ~ 2.5 min, which was reduced by polypropylene contacts (0–2 $\times$) and absent in JT010-free controls (Cont.). **E**, The mass spectrum covering the period at ~ 2.5 min shows a predominant peak of 355 m/z as expected for JT010. The mass spectrum shown in **E** refers to the period marked with * in **D**. **F**, The single ion record (SIR) of 355 m/z , averaged from five runs (100% = 2.5×10^6 counts) in water and DMSO. **G**, Area under the curve recorded for JT010 2 μM due to one and two polypropylene tip contacts in water and DMSO. *** $p < 0.001$.

Discussion

We demonstrated that an isolated and specific stimulation of TRPA1 in human skin causes pain. The established TRPA1-specific pain model is based on the potent TRPA1 agonist JT010. Specificity of the easily implementable human pain model for TRPA1 is further corroborated by complete inhibition by the TRPA1 antagonist A-967079. The model can be used to validate drug candidates inhibiting TRPA1 in humans.

Substantial species differences, in particular between mice and humans, hamper translation of preclinical to clinical results regarding TRPA1 function (Xiao et al., 2008; Cordero-Morales et al., 2011). Previous studies of TRPA1-related human pain models investigated at best TRPA1 among other targets, mainly due to the lack of a selective agonist, where specificity can hardly be claimed. AITC, for instance, was injected in 1–9 M (10–90%) concentration in psychophysical experiments (Andersen et al., 2017). Concentrations >100 μ M, however, were found to activate TRPV1 as well, and at higher concentrations in the millimolar range the activation of TRPV1 exceeds TRPA1 by many times, as the latter can even be inhibited again (Everaerts et al., 2011; Gees et al., 2013; Meseguer et al., 2014). For example, when AITC 10 mM was injected less than half of the behavioral responses was blocked by TRPA1 inhibition (Salaat and Filipek, 2015).

Similarly, cinnamaldehyde was applied topically in a concentration of ≥ 75 mM (Roberts et al., 2011), and a concentration as low as 5 mM was demonstrated to activate TRPV3 (Macpherson et al., 2006). Recently, we injected human subjects with carvacrol, which is a noncovalent agonist occurring naturally in oregano and thyme. TRPA1 antagonist A-967079 only partially (31%) inhibited the carvacrol-induced pain (Schwarz et al., 2017), which might be explained by carvacrol 500 μ M also targeting anocetamin 1 (Takayama et al., 2017). For many other substances, for example, 15- δ PGJ₂ (Cruz-Orengo et al., 2008), a TRPA1-specific mechanism has been reported in animals, but no translation to a human pain model has been attempted.

JT010-induced pain

The specificity of JT010 1 μ M for TRPA1 was established by testing a panel of TRP channels involved in nociception, including TRPV1, TRPV3, TRPV4, TRPM2, TRPM8, and TRPC5, using a concentration of 1 μ M (Takaya et al., 2015). To allow for estimation of the EC₅₀, concentrations of JT010 included 3.1 μ M. Therefore, lack of activation of TRPV1 expressed in HEK293t cells by JT010 3.1 μ M (and 10 μ M) has been demonstrated in this manuscript. The significant cubic polynomial contrast shows that at lower concentrations an increase of JT010 pain was associated with increased pain, but this levels off at higher JT010 concentrations. This might be indicative that a concentration of 3.1 μ M JT010 is above the EC₅₀, allowing the estimation of the latter.

Capsaicin was used as a test stimulus in a training session in all subjects. As observed in cellular experiments, the onset of pain was faster for capsaicin compared with JT010. This is probably explained by the slower chemical activation, including covalent modification of TRPA1 (Hinman et al., 2006; Macpherson et al., 2007; Bahia et al., 2016). The intradermal injection model caused a short pain sensation, which showed good tolerability in volunteers. This is also supported by completion of the study by all recruited subjects.

In our study, the pain perception profile of capsaicin and JT010 was largely identical, generating a primarily burning pain sensation. TRPA1 was originally proposed to be a noxious cold sensor, a notion that turned out controversial. The human subjects in the present study did not report cold or hot perception.

Our results suggest that it cannot be excluded that TRPA1 contributes to cold perception, but an isolated TRPA1 activation is at least not sufficient to generate cold pain.

JT010 stability

Compared with pilot experiments for protocol optimization, during the first run of the study all samples were generated at one point in time simultaneously, and included several contacts to polypropylene surfaces. In addition, preparation, including blinding, took substantially longer, which might further explain the estimated loss in actually injected JT010 concentration. Adsorption, shown by substance recovery after a single exposure and by desorption with a lipophilic solvent, is a general problem of lipophilic compounds, and therefore may apply to other TRPA1 agonists. We observed a surface-dependent adsorption of a considerable amount of JT010. This can be expected to be concentration dependent, with a higher fraction being adsorbed to polypropylene for lower substance concentrations. Without investigating this in detail, comparison of the higher loss of JT010 2 μ M compared with 10 μ M supports this assumption (Fig. 9, compare G, C). The results do not support a chemical instability of JT010 in aqueous solution during experimentally relevant periods of time.

Several available pipette tips designed to handle lipophilic chemicals were tested and in all of these a considerable JT010 loss was observed (data not shown). Accordingly, the study suggests the handling of stocks in a lipophilic solvent and the avoiding of contact of the diluted solutions with plastic surfaces.

EC₅₀ considerations

The EC₅₀ from human psychophysics was higher than what might have been expected. Therefore, concentration–response curves were measured *in vitro* in CHO cells with stable TRPA1 expression. The automated and heated pipetting fluorescence plate reader allowed using small concentration steps over a large concentration range at 37°C. All measurements were performed 12-fold, on 3 separate experimental days and were consistent across experimental days. The EC₅₀ in cells was 47 nM at 37°C, which is substantially higher than reported previously. This was supported by a separate experiment on hTRPA1-expressing HEK293t cells, where a higher calcium influx was observed at lower temperature, a behavior fitting cold facilitation and opposed to heat-activated phenotypes. Unexpectedly, the EC₅₀ was not shifted between experiments at 25°C and 37°C. In summary, recording temperature does not explain the observed differences in EC₅₀ between the present data and those reported by Takaya et al., 2015. Further limitations of calcium-based assays as dye affinity, ceiling effects of the dynamic range, calcium buffering, and calcium elimination should be mentioned and might affect the inference of the concentration-dependent channel open probability.

The *in vitro* EC₅₀ value approximately eightfold lower than in psychophysical experiments, which is in line with the literature on other targets, but also with own experience regarding TRPA1, including carvacrol (Schwarz et al., 2017), nitroxyl (Eberhardt et al., 2014), and indirectly with reactive chemical species through aminolevulinic acid (Babes et al., 2016). It can be assumed that redistribution due to diffusion and convection rapidly reduces the injected concentration *in vivo*, and largely explains the observed differences to the *in vitro* results.

The high potency reported for JT010 motivated us to use this substance to establish a new pain model with high specificity for TRPA1. The endpoint read used in the prior study dramatically

underestimates the maximum effect, and by that overestimated the potency. Nevertheless, the determined cellular JT010 EC₅₀ of 0.047 μ M makes this one of the most potent available human TRPA1 agonists. For comparison, AITC with an EC₅₀ of 8 μ M might serve as a reference (Bianchi et al., 2012), with other potent and available substances sorted by increasing EC₅₀ as follows: PF-4840154 0.023 μ M (Ryckmans et al., 2011); polygodial 0.059 μ M (Iwasaki et al., 2009); and ASP7663 0.51 μ M (Kojima et al., 2014). Tear gas derivatives with picomolar EC₅₀ values have been reported, including the most potent dibenzo[b,f][1,4]oxazepine-4-carboxylic acid methyl ester (50 pM), but these substances are not commercially available (Gijzen et al., 2010). The specificity of JT010 with respect to other TRP channels was demonstrated for up to 10 μ M to cover and go beyond the concentration range used in this study.

Quantitative TRPA1 inhibition

In contrast to our previous study using carvacrol to stimulate TRPA1, the JT010-induced pain could be completely inhibited. In our previous manuscript, 87% of capsaicin-induced pain was inhibited by the TRPV1 antagonist BCTC (4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide). In comparison, A-967079 showed an even stronger inhibition of JT010-induced pain. This supports the assumption that JT010 1 μ M did not activate other targets that are not inhibited by A-967079. Further, and from a clinical and pharmaceutical perspective more importantly, A-967079 can be used to quantify the complete TRPA1-dependent component in a disease or in disease models. This allows evaluation of the significance of TRPA1 and the potential therapeutic benefit from targeting this receptor.

Limitations

The insertion pain was positively associated with the pain AUC. The insertion pain varies substantially between injections in every individual and is likely due to parameters, which cannot be easily assessed like the local nerve fiber density. Human volar forearm skin has been chosen mainly for practical reasons. Based on experiments with different animal tissues, we know that response characteristics are largely uniform. Consequently, it can be assumed that the results would be similar in other part of the body. Therefore, acquisition of the insertion pain can be recommended to allow for correction of this confounding factor. A further practical limitation could be considered the required substance handling for the JT010-induced pain model, complicating the usual routine processing using plasticware.

Conclusion

An easily implementable human TRPA1-specific pain model was established and thoroughly tested. The model is characterized by complete stimulus inhibition by a TRPA1 antagonist, which can serve as reference for further antagonists. Using a TRPA1 antagonist validated in a human model will allow to quantify the TRPA1-dependent component in pathophysiology and disease.

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