Repeated administration of vanilloid receptor TRPV1 antagonists attenuates hyperthermia elicited by TRPV1 blockade


Running Title: Hyperthermia attenuates after repeated dosing of AMG 517

*Address correspondence to: Dr. Narender R. Gavva, Department of Neuroscience, MS-29-2-B, One Amgen Center Drive, Thousand Oaks, CA 91320-1799. Phone: 805-447-0607, Fax: 805-480-1347; Email: ngavva@amgen.com

Total number of pages including all figures & tables: 42

Figures: 6
Tables: 1
References: 47

Total number of words in abstract: 250
Total number of words in introduction: 423
Total number of words in discussion: 1247

Abbreviations: TRPV1: transient receptor potential vanilloid type 1; TRPA1: transient receptor potential ankyrin 1; TRPM8: transient receptor potential melastatin 8; TRPV2: transient receptor potential vanilloid type 2; TRPV3: transient receptor potential vanilloid type 3; TRPV4: transient receptor potential vanilloid type 4; 2-APB: 2-Aminoethoxydiphenyl borate; 4αPDD: 4alpha-Phorbol 12,13-didecanoate; CHO: Chinese hamster ovary; CFA: complete Friend’s adjuvant; MED: minimally effective dose; MEC: minimally effective concentration; AMG 517: N-(4-[6-(4-trifluoromethyl-phenyl)-pyrimidin-4-yloxy]-benzothiazol-2-yl)-acetamide; AMG8163: tert-butyl_2-(6-([2-(acetylamino)-1,3-benzothiazol-4-yl]oxy)pyrimidin-4-yl)-5-(trifluoromethyl)phenylcarbamate; compound M8-B: N-(2-aminoethyl)-N-((3-(methyloxy)-4-((phenylmethyl)oxy)phenyl)methyl)-2-thiophenecarboxamide; AMG9810: (E)-3-(4-t-
butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide; JYL1421: N-(4-(((4-tert-
butylbenzyl)amino|carbonothioyl)amino)methyl]-2-fluorophenyl)methanesulfonamide;
compound V3-H: 1-(((5-chloro-1,3-benzothiazol-2-yl)thio)acetyl)-8-methyl-1,2,3,4-
tetrahydroquinoline; EMEA: European Agency for the Evaluation of Medicinal Products; US
FDA: United States Food and Drug Administration; CPMP: Committee for Proprietary
Medicinal Products; ICH: International Conference on Harmonisation
Abstract

Capsaicin, the active ingredient in some pain relieving creams, is an agonist of a non-selective cation channel known as the vanilloid receptor, TRPV1. The pain relieving mechanism of capsaicin includes desensitization of the channel, suggesting that TRPV1 antagonism may be a viable pain therapy approach. In agreement with the above notion, several TRPV1 antagonists have been reported to act as anti-hyperalgesics. Here, we report the in vitro and in vivo characterization of a novel and selective TRPV1 antagonist, AMG 517, and compare its pharmacology with a closely related analogue, AMG8163. Both AMG 517 and AMG8163 potently and completely antagonized capsaicin, proton, and heat activation of TRPV1 in vitro, and blocked capsaicin-induced flinch in rats in vivo. To support initial clinical investigations, AMG 517 was evaluated in a comprehensive panel of toxicology studies that included in vivo assessments in rodents, dogs, and monkeys. The toxicology studies indicated that AMG 517 was generally well tolerated; however, transient increases in body temperature (hyperthermia) were observed in all species following AMG 517 dosing. To further investigate this effect, we tested and showed that the anti-pyretic, acetaminophen, suppressed the hyperthermia caused by TRPV1 blockade. We also showed that repeated administration of TRPV1 antagonists attenuated the hyperthermia response while the efficacy in capsaicin-induced flinch model was maintained. In conclusion, these studies suggest that the transient hyperthermia elicited by TRPV1 blockade may be manageable in the development of TRPV1 antagonists as therapeutics. However, the impact of TRPV1 antagonist-induced hyperthermia on their clinical utility is still unknown.
Introduction

Pain is a mechanism of the host defense warning system transduced through small diameter nociceptors that signal on-going damage to the body. By its predominant expression in c-fibers, the vanilloid receptor, TRPV1, plays a key role in the detection of noxious painful stimuli such as acid (low pH), noxious heat, components of the “inflammatory soup,” vanilloids such as capsaicin (the pungent component of hot chili peppers), and resiniferatoxin (an alkaloid from Ephorbia resinifera) (Caterina et al., 1997; Szallasi and Blumberg, 1999; Holzer, 2004; Szolcsanyi, 2004). Capsaicin and resiniferatoxin revealed the potential of TRPV1 as a target for pain and helped to identify and clone the TRPV1 channel. Although capsaicin and resiniferatoxin cause burning pain acutely, chronic treatment was shown to produce analgesia through ablation of TRPV1 expressing neurons. These results indicated the potential of TRPV1 agonists as pain therapeutics (reviewed in (Bley, 2004)).

Up-regulation of TRPV1 has been shown in inflammatory pain models in preclinical species (Ji et al., 2002) and human disease conditions such as inflammatory bowel disease (Matthews et al., 2004), painful bladder syndromes (Mukerji et al., 2006), vulvodynia (Tympanidis et al., 2004), and chronic persistent cough (Groneberg et al., 2004). In addition, TRPV1 knockout mice showed reduced thermal hypersensitivity after inflammatory tissue injury (Caterina et al., 2000; Davis et al., 2000) and attenuated experimental arthritis (Szabo et al., 2005; Barton et al., 2006). Finally, TRPV1 antagonists (that block capsaicin, proton and heat activation) representing various chemotypes acted as anti-hyperalgesics in inflammatory (Pomonis et al., 2003; Valenzano et al., 2003; Walker et al., 2003; Gavva et al., 2005b; Honore et al., 2005; Rami et al., 2006; Gunthorpe et al., 2007), and surgical-incision (Honore et al., 2005) pain models as well as analgesics in cancer pain (Ghilardi et al., 2005) models (reviewed in (Immke and Gavva, 2006;
Szallasi et al., 2007)). Taken together, the above data generated strong interest in TRPV1 as a therapeutic target for pain.

Most of the published TRPV1 antagonists lack optimal properties for clinical development such as selectivity, solubility, oral bioavailability, and/or reasonable pharmacokinetics. After evaluation of TRPV1 antagonists representing various chemotypes (Doherty et al., 2005; Xi et al., 2005; Ognyanov et al., 2006; Doherty et al., 2007; Norman et al., 2007) and unpublished data) for potency, selectivity, plasma half-life, effects in the capsaicin-induced flinch model, and efficacy in models of inflammatory pain, we identified AMG 517 as a potential drug for the management of pain in humans. Here, we describe the characterization of AMG 517 and the data set leading to its selection as a clinical candidate.
Methods

All animal use and husbandry were in accordance with the USDA Animal Welfare Act (9 CFR, Parts 1, 2 and 3) and the Guide for the Care and Use of Laboratory Animals (ILAR publication, 1996, National Academy Press). The cDNA sequences used in this study are identical or similar to AJ277028 (human TRPV1), NP_114188 (rat TRPV1), Q704Y3 (mouse TRPV1), XP_001117609 (Rhesus TRPV1), EF100779 (Cynomolgus TRPV1), Q9WUD2 (rat TRPV2), NP659505 (human TRPV3), NP067638 (human TRPV4), O75762 (human TRPA1) and NP_076985 (human TRPM8).

Agonist-induced $^{45}$Ca$^{2+}$ uptake assay. Two days prior to the assay, cells were seeded in Cytostar 96 well plates (Amersham) at a density of 20,000 cells/well. The activation of TRPV1 and TRPV2 was followed as a function of cellular uptake of radioactive calcium ($^{45}$Ca$^{2+}$, ICN). Capsaicin (0.5 µM), pH 5, and heat (45 °C) were used as agonists for TRPV1 and 2-APB (200 µM) was used as agonist for TRPV2. All the antagonist $^{45}$Ca$^{2+}$ uptake assays were conducted as reported previously (Gavva et al., 2005b) and had a final $^{45}$Ca$^{2+}$ concentration of 10 µCi/mL. Radioactivity was measured using a MicroBeta Jet (Perkin-Elmer Inc.). Data were analyzed using Graphpad Prism 4.01 (GraphPad Software Inc, San Diego, CA).

Luminescence readout assay for measuring intracellular calcium. Stable CHO cell lines expressing human TRPA1, TRPM8, TRPV3, and TRPV4 were generated using tetracycline inducible T-REx™ expression system from Invitrogen, Inc (Carlsbad, CA). In order to enable a luminescence readout based on intracellular increase in calcium (Le Poul et al., 2002), each cell line was also co-transfected with pcDNA3.1 plasmid containing jelly fish aequorin cDNA. Twenty four hours before the assay, cells were seeded in 96-well plates and TRP channel
expression was induced with 0.5 µg/ml tetracycline. On the day of the assay, culture media was removed and cells were incubated with assay buffer (F12 containing 30 mM HEPES for TRPA1, TRPM8, and TRPV3; F12 containing 30 mM HEPES, 1 mM CaCl₂, and 0.3% BSA for TRPV4) containing 15 µM coelenterazine (P.J.K, Germany) for 2 hours. Antagonists were added for 2.5 min prior to addition of an agonist. The luminescence was measured by a CCD camera based FLASH-luminometer built by Amgen, Inc. Following agonists were used to activate TRP channels: 80 µM AITC for TRPA1, 1 µM icilin for TRPM8, 200 µM 2-APB for TRPV3, and 1 µM 4α-PDD for TRPV4. Compound activity was calculated using GraphPad Prism 4.01 (GraphPad Software Inc, San Diego, CA).

Capsaicin-induced flinch model. Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 190-220 g were allowed at least 3 days of acclimation in Amgen’s Association for Assessment and Accreditation of Laboratory Animal Care approved-animal care facility prior to the start of the experiment. Animals (8 per group) were pretreated with vehicle (a dose volume of 5 ml/kg in an Ora-Plus/5% Tween-80) or TRPV1 antagonists (by oral gavage), 2 hours prior to intraplantar injection of 0.5 mg of capsaicin in a volume of 25 µl of 5% EtOH in phosphate-buffered saline without Ca²⁺ and Mg²⁺ (Sigma-Aldrich Inc., St. Louis, MO). Immediately following the injection of capsaicin, the number of flinches was recorded over a 1-minute period by an individual fully blinded to treatment conditions. Blood samples were collected immediately following behavioral testing for pharmacokinetic (PK) analyses.

Radio telemetry in rats. Male Sprague Dawley rats (CRL, Wilmington, MA) weighing 175-250 g (6 – 8 weeks of age) were single-housed and allowed at least a 1-week acclimation period in
prior to the start of the experiment. The temperature in the room used for the experiments was maintained at 20 ± 2 °C. Rats were implanted with a radiotelemetry probe (model ER-4000 PDT, Mini Mitter, Brend, OR) and allowed at least 3 days of recovery prior to the drug experiment. On the day of the experiment, single-housed animals were placed on radiotelemetry receivers. Baseline body core temperature was recorded for 30 min prior to treatment. Rats (8 per group) were treated with vehicle (a dose volume of 5 ml/kg in an Ora-Plus/5% Tween-80) or TRPV1 antagonists (by oral gavage) with continuation of body core temperature recording for 2 hours post-drug administration. Blood samples were collected immediately following behavioral testing for PK analyses.

**CFA-induced thermal hyperalgesia.** After multiple days of full habituation to the testing equipment and paradigm, CFA-induced thermal hyperalgesia was evaluated by measuring paw withdrawal latencies in male Sprague Dawley rats. Twenty-one hours after CFA injection (50 µl of 0.1%), animals were dosed (p.o.) with AMG 517 or AMG8163 at a dose range of 0.001 to 30 mg/kg in a volume of 5 ml/kg. Two hours after drug dosing (23 hours after CFA injection), paw withdrawal latencies were measured using modified Hargreaves hot boxes (UCSD, CA) by investigators fully blinded to treatment conditions.

**Body temperature evaluation in radiotelemetred cynomolgus monkeys treated with single doses of AMG 517.** Six experimentally non-naïve Cynomolgus monkeys (3 males and 3 females) weighing 2.6 to 4.8 kg were used on the study. Animals had surgically implanted radiotelemetry devices (Model Number TL11M2-D70-PCT, Data Sciences™ International, St. Paul, Minnesota) and were housed individually in stainless-steel cages.
Groups 1 and 2 were treated with a single dose of vehicle (10% Pluronic F108 in Ora-Plus™) via nasogastric gavage and observed for 7 days post-dose. Subsequently, Group 1 was treated with a single dose of 10 mg/kg AMG 517 and Group 2 was treated with a single dose of 30 mg/kg AMG 517; both groups were observed for 7 days post-dose. Individual body temperatures were monitored by telemetry using the Data Sciences™ computer system (Dataquest® ART Gold data acquisition and analysis software). Body temperature data were recorded from each animal for 30 seconds at 5-minute intervals prior to, during, and following dosing beginning approximately 60 minutes before the initiation of dosing and continuing through 72 hours post-dose, and for 30 seconds at hourly intervals from 72 hours through 165 hours post-dose. Mean body temperature across each 30-second recording period was calculated by the Dataquest® software.

For pharmacokinetic analyses, blood samples were collected from all animals at pre-dose, and approximately 1, 2, 4, 24, 72, and 168 hours after dosing. AMG 517 levels were quantitated in lithium-heparinized plasma samples utilizing a 96-well protein precipitation with a reversed phase LC-MS/MS analytical procedure. Individual plasma concentration-time data were analyzed by non-compartmental methods using WinNonlin™ (Pharsight Corporation, Mountain View, CA). After the last data collection, all animals were returned to the colony.

Body temperature evaluation in naïve cynomolgus monkeys treated with repeated daily doses of AMG 517. Cynomolgus monkeys (6/sex/group), weighing 2.0 to 3.4 kg, were treated by nasogastric gavage with 0 (vehicle control; 10% Pluronic F108 in Ora-Plus™), 30, 100, or 500 mg/kg/day AMG 517 for 28 consecutive days (5 mL/kg/day). Rectal body temperatures were
collected prior to dosing, and at 3 and 24 hours after dosing on Days 1, 8, 15, 22, and 28 of treatment. Mean body temperatures at each timepoint were compared across groups with a 1-way analysis of variance (ANOVA) utilizing $p \leq 0.05$ as the level of significance. If the ANOVA was significant, Dunnett's multiple comparison test was used to identify statistically significant differences between each drug-treated group and the vehicle-treated control group (at $p \leq 0.05$).
Results

Characterization of AMG 517 as a TRPV1 antagonist

In our efforts to identify a TRPV1 antagonist clinical candidate, we synthesized several novel series of molecules representing various chemotypes ((Doherty et al., 2005; Xi et al., 2005; Ognyanov et al., 2006; Doherty et al., 2007; Norman et al., 2007); unpublished data) and evaluated their ability to block various modes of TRPV1 activation. AMG 517 inhibited capsaicin, pH 5, and heat induced \(^{45}\)Ca\(^{2+}\) uptake into cells expressing TRPV1 with IC\(_{50}\) values of 1-2 nM (Table 1, Fig.1A). AMG 517 blocked capsaicin-, proton-, and heat-induced inward currents in TRPV1 expressing cells similarly (data not shown). In addition, AMG 517 acted as a potent antagonist of all of the endogenous ligands tested (Table 1). AMG 517 inhibited native TRPV1 activation by capsaicin in rat dorsal root ganglion neurons with an IC\(_{50}\) value of 0.68 \(\pm\) 0.2 nM. AMG 517 is a competitive antagonist of both rat and human TRPV1 with dissociation constant (K\(_{b}\)) values of 4.2 and 6.2 nM, respectively (Fig. 1B-E). AMG 517 did not activate TRPV1 at concentrations up to 40 \(\mu\)M, as measured by \(^{45}\)Ca\(^{2+}\) uptake into TRPV1 expressing cells, indicating that it was not a partial agonist.

Pharmacology of another TRPV1 antagonist, AMG8163 (a ‘\(\tau\)-butyloxycarbonyl’ analogue of AMG 517), used in this study was reported recently (Magal, 2005; Gavva et al., 2007).

AMG 517 was found to be selective for TRPV1 among the recombinant TRP family members that we have tested (Fig.2A-E). The IC\(_{50}\) value for AMG 517 was >20 \(\mu\)M against 2-APB activated TRPV2 and TRPV3, 4-\(\alpha\)PDD activated TRPV4, allyl isothiocyanate activated TRPA1, and icilin activated TRPM8 in cell based assays that measure agonist-induced increases in intracellular calcium in CHO cells recombinantly expressing the appropriate TRP channel. In a selectivity screen conducted by Novascreen\textsuperscript{\textregistered}, 10 \(\mu\)M concentration of AMG 517 showed
significant binding (≥ 45% inhibition) to only 3 of 90 evaluated targets that included receptors, enzymes, and ion channels (Supplementary information). AMG 517 showed binding to peripheral monoamine oxidase-B (88%) and site 2 of the sodium ion channel (47%), and showed 79% inhibition of anandamide transport in a functional assay. IC₅₀ values of 3.4, 11.3, and 19.4 µM were subsequently determined for the monoamine oxidase-B (peripheral), anandamide transporter, and sodium channel (site 2) targets, respectively.

Pharmacokinetic analyses showed that plasma half-life values of AMG 517 were 31, 41, and 62 hrs in rats, dogs, and monkeys, respectively. Oral bioavailability was 47-51% in rats, 23-29% in dogs, and 42-52% in monkeys (Doherty et al., 2007).

TRPV1 antagonists block capsaicin-induced flinch in rats

Since the pharmacokinetic properties of AMG 517 and its closely related analogue, AMG8163, were amenable to in vivo studies, they were evaluated in a rat capsaicin-induced flinch model as a measure of TRPV1 antagonism (Seabrook et al., 2002). While oral administration of both AMG 517 and AMG8163 produced a dose-dependent increase in plasma concentrations, they also produced a dose-dependent decrease in the number of flinches induced by capsaicin treatment (Fig. 3A,B and (Doherty et al., 2007; Gavva et al., 2007). The minimally effective dose (MED), based on a statistically significant difference in number of flinches from the vehicle versus capsaicin administered group, was 0.3 mg/kg (p < 0.05) for AMG 517 and 0.03 mg/kg (p < 0.05) for AMG8163. The corresponding plasma concentrations were 90-100 ng/ml for AMG 517 and approximately 10 ng/ml for AMG8163. Correlating with its long plasma half-life in rats, the time course of AMG 517 (3 mg/kg) exhibited significant reductions in capsaicin-induced flinch up to 24 hrs after dosing (Fig. 3C). At these time points, plasma levels of AMG
517 were \( \geq 300 \) ng/ml. There was no difference in flinch response between the vehicle group and groups treated with AMG 517 by 48 hrs.

To evaluate the effect of repeated dosing with TRPV1 antagonists on capsaicin-induced flinch, 3 mg/kg of AMG8163 was administered twice (p.o.) daily for 4 days. Complete inhibition of the capsaicin-induced flinch response on days 2, 3, and 4 indicated continuous antagonism of TRPV1 by AMG8163 \textit{in vivo} (Fig. 3D).

TRPV1 antagonists act as anti-hyperalgesics in rat models of inflammatory pain and cause hyperthermia in rats

Next, we evaluated the ability of AMG 517 and AMG8163 to block pain behavior (thermal hyperalgesia) under inflammatory conditions in rats. Intra-plantar injections of complete Freund's adjuvant (CFA) produced a dose-dependent thermal hyperalgesia indicated by a decrease in paw withdrawal latency in rats, 24 hrs post-injection. TRPV1 antagonists such as AMG9810, A-425619, and BCTC were shown to block hyperalgesia induced by CFA (Pomonis et al., 2003; Gavva et al., 2005b; Honore et al., 2005). As expected, oral administration of both AMG 517 and AMG8163 reversed established thermal hyperalgesia in a dose-dependent manner at 21 hrs after CFA injection (Fig. 4A,B; (Doherty et al., 2007)). Associated plasma concentrations increased in a dose-dependent manner for both drugs up to 10 mg/kg. The minimally effective dose (MED) was 1 mg/kg (p.o.) for AMG 517 and 0.3 mg/kg (p.o.) for AMG8163. The or minimally effective concentration (MEC) was approximately 300 ng/ml in plasma for AMG 517 (with a maximal reversal of ~40%) and 10 - 30 ng/ml in plasma for AMG8163 (with a maximal reversal of ~50%). In the same experiment, the positive control, ibuprofen (30 mg/kg, p.o.), produced a comparable (~55%) reversal of CFA-induced thermal
hyperalgesia. In addition, TRPV1 antagonists showed efficacy in carrageenan-induced (42 % reversal by 3 mg/kg dose of AMG 517) and surgical incision-induced (48 % reversal by 3 mg/kg dose of AMG8163) thermal hyperalgesia (Supplementary Fig.1). Since most TRPV1 antagonists reported to-date, including AMG 517 and AMG8163, showed only 40-50% block of hyperalgesia in rodent models of pain (reviewed in (Immke and Gavva, 2006)), we concluded that TRPV1 partially contributes to inflammation- and incision-induced hyperalgesia.

We and others have previously shown that TRPV1 antagonists cause transient hyperthermia in multiple species (rats, dogs, and cynomolgus monkeys). These observations suggested that TRPV1 is tonically activated in naïve rats, dogs, and primates, and that tonic TRPV1 activation regulates body temperature (Bannon, 2004; Swanson et al., 2005; Gavva et al., 2007). As expected, AMG 517 caused transient hyperthermia in rodents, dogs, and monkeys (Fig. 4C,D; data not shown). AMG 517 induced hyperthermia in a steep dose dependent manner, with 0.3, 1, and 3 mg/kg associated with 0.5 °C, 0.6 °C, and 1.6 °C increases in body temperature, respectively. Body temperatures of rats treated with all doses of AMG 517 returned to baseline within 10 to 20 hrs. In rats, body temperatures in the vehicle group varied from 37.4 °C ± 0.05 °C during the light cycle to 37.8 °C ± 0.05 °C during the dark cycle, suggesting that body temperature is a dynamically regulated process. In monkeys, single doses of AMG 517 at 10 and 30 mg/kg caused an approximate 1 to 1.5 °C increase in body temperature that lasted beyond 24 hours. Mean temperatures in both drug-treated groups differed from the vehicle treated group in a statistically significant manner (ANOVA/Dunnett’s) at the majority of evaluated post-dose time-points (Fig.4D).

Toxicological evaluation of AMG 517
To support initial clinical investigations, AMG 517 was evaluated in a package of non-clinical toxicology studies that was designed in accordance with relevant regulatory guidelines (ICH, 2000). Pivotal toxicology studies were conducted in compliance with global Good Laboratory Practice (GLP) regulations, including those defined by the US FDA (21 CFR Part 58). Individual studies were designed in consideration of relevant regulatory guidelines (refer to cited (selected) guidelines for detail). The package included single dose studies in mice and rats (US-FDA, 1996), in vitro and in vivo safety pharmacology studies (ICH, 2001), repeated dose studies in rats, monkeys, and dogs (ICH, 1995; EMEA-CPMP, 2000), and a standard battery of in vitro and in vivo genetic toxicity studies (ICH, 1997). AMG 517 was administered p.o. in all in vivo studies. Definitive 28-day repeated-dose studies evaluated daily oral doses up to 500 mg/kg/day in rats and monkeys, producing end-of-treatment C_{max} levels of 13,500 ng/mL and 9,160 ng/mL, respectively, at 500 mg/kg/day. Notably, no AMG 517-related adverse effects were observed in any of the above-listed toxicology studies. However, transient drug-related body temperature increases (see below) were noted in mice, rats, dogs, and monkeys.

TRPV1 antagonist-induced hyperthermia attenuates after repeated dosing

To evaluate the effects of repeated dosing on TRPV1 antagonist-induced hyperthermia, we administered AMG8163 twice daily for 4 days to rats implanted with radiotelemetry probes and monitored their body temperature (Fig. 5A). Since body temperature measurements were gathered by continuous monitoring we compared the effect of AMG8163 on subsequent days by averaging the temperature from each treatment group for each 24 hour period and ran 4 separate t-tests comparing the AMG8163 treated group to the vehicle treated group. AMG8163 induced a 0.5 to 0.8 °C increase in body temperature on the first day of treatment that was not apparent by
day 4 (Fig. 5A,B), suggesting that TRPV1 antagonist-induced hyperthermia attenuates with repeat dosing. Statistically significant differences ($F_{11} = 2.9; p<0.05$) were only apparent on day 1, with average temperature in the vehicle treated group of $37.6 \degree C \pm 0.12 \degree C$ and average temperature in the AMG8163 treated group equal to $38.1 \degree C \pm 0.07 \degree C$ (Fig. 5B).

AMG 517 produced an approximate $1 \degree C$ increase in body temperature in both rats and monkeys (Fig. 4C,D and data not shown). When administered as a single dose, the post-treatment temperature increase was transient, peaking within a few hours and lasting up to 20 hrs. The AMG 517-induced hyperthermic response diminished with repeat dosing in rats (data not shown), dogs (data not shown), and monkeys (Fig. 5C), as seen with AMG8163 in rats (Fig. 5A,B). In monkeys treated daily for 28 consecutive days, transient increases (up to approximately $1.1 \degree C$ greater than controls) in mean body temperature, relative to pre-dose, were observed on Day 1 in all drug treated groups (30, 100, 500 mg/kg/day), with mean temperatures at 3 hours post-dose statistically significantly ($p < 0.05$) higher than those in the control group. Following continued repeated dosing, clear drug-related patterns in an effect on body temperature (on days 8, 15, 22, and 28 of the treatment period) were not readily apparent (no other statistically significant differences from controls were observed). This observation suggests that a physiological tolerance develops with repeat dosing in rodents, canines, and non-human primates.

TRPV1 antagonist-induced hyperthermia can be suppressed by anti-pyretic acetaminophen

Because we predicted that TRPV1 antagonists might produce hyperthermia in humans, we tested whether anti-pyretic acetaminophen could suppress TRPV1 antagonist-induced hyperthermia. We administered acetaminophen, before and after AMG8163 administration, to rats surgically

17
implanted with radiotelemetry probes. Acetaminophen (300 mg/kg) suppressed the established TRPV1 antagonist-induced hyperthermia, as evidenced by a drop of body temperature from 38.5 °C to 37 °C (**Fig. 6A,B**). During baseline telemetry recordings, there was no significant difference among groups ($F_{3,8} = 3.5$, $p>0.05$) with an average temperature across groups of 36.8 °C ± 0.03 °C. AMG8163-treated rats showed an average temperature of 38.0 °C ± 0.02 °C, while the average temperature in vehicle-treated rats was 37.2 °C ± 0.03 °C, during the 30 to 120 min post dosing phase ($t_{298} = 21.2$, $p<0.01$). During the 30 to 60 min following acetaminophen administration, average body temperature dropped to 36.4 °C ± 0.04 °C, while the vehicle treated group showed an average temperature of 37.2 °C ± 0.03 °C ($t_{148} = 18.3$; $p<0.01$) (**Fig. 6B**). Acetaminophen reduced the AMG8163-induced body temperature to 37.3 °C ± 0.05 °C, while rats treated with AMG8163 alone had an average temperature of 37.8 °C ± 0.03 °C ($t_{147} = 9.1$; $p<0.01$). This average temperature in acetaminophen/AMG8163 treated rats temperature is within 0.1 °C of the vehicle-treated group’s average temperature (not statistically significant; $t_{148} = 1.5$; $p>0.05$), but approximately 0.5 °C greater than the average temperature in the vehicle/acetaminophen treated group ($t_{158} = 15.4$; $p<0.01$) (**Fig. 6B**). Low doses of acetaminophen (100 or 150 mg/kg) that did not drop body temperature by themselves were not effective in reversing AMG8163-induced hyperthermia (data not shown). Acetaminophen’s (**Fig. 6A,B**) reversal of AMG8163-induced hyperthermia suggests that TRPV1 antagonist-induced hyperthermia could potentially be managed in humans with anti-pyretics, if required.
Discussion

In the present study, we characterized AMG 517 as a potent TRPV1 antagonist and compared its pharmacology with a closely related analogue, AMG8163, in various in vitro and in vivo assays. Both molecules are potent antagonists of TRPV1 activation by chemical ligands (capsaicin, proposed endogenous agonists, and protons) and heat. Since both AMG 517 and AMG8163 bind the same pocket as capsaicin and block all modes of TRPV1 activation, they represent ‘group A’ antagonists (Gavva et al., 2005a) and data not shown) that lock the channel conformation in a ‘closed state.’ We demonstrated the ability of AMG 517 and AMG8163 to block TRPV1 in vivo by showing antagonism of capsaicin-induced flinch in rats and their anti-hyperalgesic properties through reduction of thermal hyperalgesia in inflammatory pain models (this study and Doherty et al., 2007). Based on its pharmacological activity, favorable pharmacokinetic properties (Doherty et al., 2007), and favorable non-clinical safety profile in toxicology studies, AMG 517 was chosen for clinical development.

We and others previously showed that TRPV1 antagonists that act as anti-hyperalgesics can cause transient hyperthermia in multiple species (Bannon, 2004; Gavva et al., 2007) (Swanson et al., 2005). As expected, AMG 517 caused hyperthermia in rodents, dogs, and monkeys. Importantly, as little as 0.3 mg/kg AMG 517 (MED) or its associated 100 ng/ml plasma concentration (MEC), induced transient hyperthermia. Notably, this hyperthermia-inducing MEC/MED is comparable to the MEC/MED required to demonstrate on-target coverage in the capsaicin-induced flinch model. The MEC/MED required for anti-hyperalgesia in CFA-induced pain model is higher for both antagonists compared to the MEC/MED for causing hyperthermia. This suggests that antagonism of a small pool of tonic TRPV1 activation is sufficient to trigger hyperthermia, whereas antagonism of a large pool of TRPV1 is required.
for anti-hyperalgesia in models of inflammation. Since AMG 517 is a selective TRPV1 antagonist (IC$_{50}$ against closely related TRP channels is >5,000-fold), the hyperthermia and anti-hyperalgesia were believed to be caused by TRPV1 antagonism alone. Both AMG 517 and AMG8163 are brain penetrant compounds with brain/plasma ratios of 1.01 and 0.58, respectively. As implicated recently (Cui et al., 2006), the site of action for anti-hyperalgesia by TRPV1 antagonists may be present in the central nervous system, thus requiring higher brain concentrations of antagonists for significant effects.

Although TRPV1 agonists such as capsaicin and resiniferatoxin cause a drop in body temperature (hypothermia), until recently it was not known if TRPV1 is tonically activated in vivo or even if it is involved in body temperature regulation. We recently demonstrated that TRPV1 is tonically activated in vivo based on the fact that antagonists representing a wide variety of chemotypes that block TRPV1 activation by capsaicin, heat, and protons (as well as those that block only capsaicin and heat activation), caused transient hyperthermia through a site of action outside of the blood-brain barrier (Gavva et al., 2007). Selective antagonists of TRPV1 that cause hyperthermia in multiple species (AMG9810, AMG8163 and several others in rats, JYL1421 in dogs and monkeys (Gavva et al., 2007); or JNJ-17203212 in rats (Swanson et al., 2005) and AMG 517 in multiple species [this study]) further demonstrate that TRPV1 is tonically activated in vivo and is involved in body temperature regulation. The fact that AMG 517 caused transient hyperthermia in rats, dogs, and monkeys, further confirms that the involvement of TRPV1 in thermoregulation is conserved across rodents to primates.

The mechanisms of TRPV1 agonist induced hypothermia include skin vasodilation and reduction in metabolic heat production (reviewed in (Hori, 1984)); whereas the mechanisms of TRPV1 blockade elicited hyperthermia include skin vasoconstriction that reduces heat loss and
an increase in metabolic heat production as measured by increased oxygen consumption in rats (Steiner et al., 2007). In addition, agonist-induced hypothermia and antagonist-induced hyperthermia are absent in TRPV1 knockout mice (Caterina et al., 2000; Steiner et al., 2007), suggesting that the effects of agonists and antagonists on body temperature are exclusively mediated by TRPV1 channels. Even though TRPV1 is activated by heat, it is not known if TRPV1 itself acts as a thermosensor for the body. It appears that TRPV1 blockade does not affect the thermosensation because thermal preference of TRPV1 antagonist-administered rats was not altered in a thermotaxis gradient assay (Steiner et al., 2007), suggesting that TRPV1 may not act as a thermosensor \textit{in vivo}. Interestingly, TRPV3 knockout mice display strong deficits in responses to innocuous and noxious heat in thermotaxis assays, suggesting that TRPV3 acts as a thermosensor (Moqrich et al., 2005; Dhaka et al., 2006).

Because body temperature maintenance is a tightly regulated physiological process, which may have multiple mechanisms to bring the body temperature towards normal range in the event of a significant change (a drop or an increase), we evaluated whether hyperthermia persists with continuous blockade of TRPV1 by repeatedly administering TRPV1 antagonists (to maintain a constant plasma concentration). Since the hyperthermia elicited by TRPV1 blockade attenuated by day 2 to 4 of repeated administration of antagonists, we hypothesize that other mechanisms suppressed TRPV1 blockade elicited hyperthermia. Both AMG 517 and AMG8163 blocked capsaicin-induced flinch response in rats during this period, demonstrated continuous blockade of TRPV1 \textit{in vivo}. We believe that other mechanisms may have compensated for the role of TRPV1 in body temperature maintenance during days 2 through 4. Similar attenuation of TRPV1 blockade elicited hyperthermia after repeated administration of another TRPV1
antagonist was recently reported (Cortright D, 2006, Spring Pain Research Conference, Apr 22-29, Grand Cayman, BWI).

Since TRPV1 antagonist-induced hyperthermia is an undesirable on-target effect, we evaluated the potential utility of antipyretics in managing such hyperthermia. We found that the antipyretic, acetaminophen, blocked development of hyperthermia when given before TRPV1 antagonists, and reversed ongoing hyperthermia when given after TRPV1 antagonists. This suggests that antipyretics might be effective clinically should TRPV1 antagonists cause hyperthermia in humans. The antipyretic reduction of hyperthermia is not mediated by a direct modulation of TRPV1, because acetaminophen neither blocks nor activates the TRPV1 channel (unpublished data). It should be noted here that doses of acetaminophen (100 or 150 mg/kg) that do not drop body temperature by themselves did not block or reverse the TRPV1 antagonist-induced hyperthermia. It was reported that TRPV1 antagonists cause the same magnitude of hyperthermia in normal rats as well as in rats with ongoing inflammation, suggesting that inflammation-induced fever and TRPV1 antagonist-induced hyperthermia act only additively (Cortright D, 2006, Spring Pain Research Conference, Apr 22-29, Grand Cayman, BWI). Consequently, antagonist-induced hyperthermia may pose an issue for clinical development of TRPV1 antagonists as therapeutics in human subjects prone to infections if temperature increases of 2-3 °C result.

Among the results presented here and elsewhere (by Cortright D, 2006, Spring Pain Research Conference, Apr 22-29, Grand Cayman, BWI), the most promising is the apparent attenuation of antagonist-induced hyperthermia with repeated dosing, suggesting that TRPV1 blockade elicited hyperthermia may be overcome. Since peripherally restricted TRPV1 antagonists cause hyperthermia (Gavva et al., 2007) and CNS penetrant antagonists seem to be
more effective anti-hyperalgesics than peripherally-restricted compounds (Cui et al., 2006), the best option seems to be the repeated-dosing mediated attenuation of TRPV1 antagonist-induced hyperthermia. Hence, carefully planned repeated dosing paradigms of TRPV1 antagonists in humans, to attenuate hyperthermia, will be a critical and necessary step for developing them as therapeutics. Still to be determined are: a) whether TRPV1 antagonists will be efficacious in human disease, b) if TRPV1 antagonists affect human body core temperature, and c) if TRPV1 blockade elicited hyperthermia will be more pronounced during infections or injuries. The impact of TRPV1 antagonist-induced hyperthermia on their clinical utility is still unclear.

Acknowledgments

The authors would like to thank Shoushu Jiao for coordinating some of the in vivo studies, Marian Stec and Partha Chakrabarti for AMG8163 synthesis, and Ning Chen for AMG 517 synthesis.
References

(2004) Involvement of TRPV1 in the regulation of body temperature in rats and mice, in
*Society for Neuroscience Annual Meeting*, Washington, DC.

Barton NJ, McQueen DS, Thomson D, Gauldie SD, Wilson AW, Salter DM and Chessell IP


type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. *J Pharmacol Exp Ther* **314**:410-421.


Szallasi A and Blumberg PM (1999) Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacological Reviews.* **51**:159-212.


carboxamides as vanilloid receptor 1 (TRPV1) antagonists. *Bioorg Med Chem Lett*

**15**:5211-5217.
Legends for Figures

Figure 1. AMG 517 is a potent and selective antagonist of TRPV1 activation. (A) Chemical structure of AMG 517. (B) AMG 517 antagonism of capsaicin activation of rat TRPV1 is surmountable. Concentration-response curves for capsaicin were generated in the absence or presence of 4, 12, 37, 111 or 333 nM AMG 517. (C) Schild analysis of data in B. (D) AMG 517 antagonism of capsaicin activation of human TRPV1 is surmountable. Concentration-response curves for capsaicin were generated in the absence or presence of 4, 12, 37, 111, 333 or 1000 nM AMG 517. (E) Schild analysis of data in D. Each point in the graph is an average ± SD of an experiment conducted in triplicate.

Figure 2. Effect of AMG 517 on the activation of different TRP channels. (A) Effect of AMG 517 on 2-APB-induced $^{45}$Ca$^{2+}$ uptake into CHO cells expressing rat TRPV2. IC$_{50}$ value of the positive control, ruthenium red was 200 nM in this assay. (B) Effect of AMG 517 on 2-APB-induced increase in intracellular calcium in CHO cells expressing human TRPV3 measured in an aequorin-readout assay. IC$_{50}$ value of the positive control antagonist, compound V3-H (1-(((5-chloro-1,3-benzothiazol-2-yl)thio)acetyl)-8-methyl-1,2,3,4-tetrahydroquinoline) was 72 nM in this assay. (C) Effect of AMG 517 on 4-αPDD-induced increase in intracellular calcium in CHO cells expressing human TRPV4 measured in an aequorin-readout assay. IC$_{50}$ value of the positive control, ruthenium red was 24 nM in this assay. (D) Effect of AMG 517 on allyl isothiocyanate-induced increase in intracellular calcium in CHO cells expressing human TRPA1 measured in an aequorin-readout assay. IC$_{50}$ values of the positive control, ruthenium red was 367. (E) Effect of AMG 517 on icilin-induced increase in intracellular calcium in CHO cells
expressing human TRPM8 measured in an aequorin-readout assay. IC₅₀ value of a positive control antagonist, compound M8-B (N-(2-aminoethyl)-N-((3-(methyloxy)-4-((phenylmethyl)oxy)phenyl)methyl)-2-thiophenecarboxamide) was 5 nM in this assay.

Figure 3. AMG 517 and AMG8163 block capsaicin-induced flinch in rats. (A) Different doses (0.003 to 3 mg/kg) of AMG 517 were administered p.o. to rats 60 min prior to capsaicin challenge. Number of flinches in the first 1 min was counted and plotted against the dose of AMG 517. Asterisks indicate p < 0.05 compared with vehicle-treated controls (ANOVA with Dunnett’s post-hoc test). Plasma concentration of AMG 517 for each dose is shown in blue bars. Parts of these results were also shown in a chemistry manuscript that describes structure-activity relationship of AMG 517 analogues (Doherty et al, 2007) (B) Different doses (0.01 to 3 mg/kg) of AMG8163 were administered p.o. to rats 60 min prior to capsaicin challenge. Number of flinches in the first 1 min was counted and plotted against the dose of AMG8163. Double asterisks indicate p < 0.01 compared with vehicle-treated controls (ANOVA with Dunnett’s post-hoc test). Plasma concentration of AMG8163 for each dose is shown in blue bars. (C) Single oral administration of AMG 517 (3 mg/kg) blocks capsaicin-induced flinch for 24 hrs. Capsaicin-induced flinch was tested at 0 time point (two hours post-dosing of AMG 517), 6 hr time point (8 hrs post dosing) and 24 hour time point (26 hrs post dosing). Asterisks indicate p < 0.05 compared with vehicle at the same time point (ANOVA with Dunnett’s post-hoc test). (D) Effect of once daily dosing of AMG8163 (3 mg/kg) on capsaicin-induced flinch response at day 2, 3 and 4. Capsaicin-induced flinch response is completely inhibited on days 2-4.
Figure 4. AMG 517 and AMG8163 block thermal hyperalgesia in CFA model of pain. (A) Significant reversal of CFA-induced thermal hyperalgesia was observed at doses ≥ 1 mg/kg of AMG 517. Double asterisks indicate p < 0.01 compared with vehicle-treated controls (ANOVA with Dunnett’s post-hoc test). Dose dependent increase in plasma concentrations are shown in blue bars. Parts of these results were also shown in a chemistry manuscript that describes structure-activity relationship of AMG 517 analogues (Doherty et al, 2007) (B) Significant reversal of CFA-induced thermal hyperalgesia was observed at doses ≥ 0.3 mg/kg of AMG8163. Double asterisks indicate p < 0.01 compared with vehicle-treated controls (ANOVA with Dunnett’s post-hoc test). Dose dependent increase in plasma concentrations are shown in blue bars. AMG 517 causes hyperthermia in rats. (C) Body temperature rats administered with either vehicle or different doses of AMG 517. A significant increase in body temperature was seen 30 to 40 min after AMG 517 administration. (D) Single doses of AMG 517 cause hyperthermia in monkeys that lasts beyond 24 hours. Mean temperatures in both drug-treated groups differed from the vehicle treated group in a statistically significant manner at the majority of evaluated post-dose time-points.

Figure 5. (A) AMG8163-induced hyperthermia attenuates after repeat dosing in rats. AMG8163 was administered twice daily (red) caused an increase of 0.5 to 0.8°C on day 1 but not on days 2, 3 and 4. Please note the diurnal temperature variation. (B) AMG8163-induced hyperthermia attenuates after repeat dosing in rats. AMG8163 caused an increase of 0.5 to 0.8°C on day 1 but not on days 2, 3 and 4. Asterisks indicate p < 0.05 compared with
vehicle-treated controls (ANOVA with Dunnett’s post-hoc test). Each bar represents daily average ± SD of body temperature in (A). (C) AMG 517-induced hyperthermia attenuates after repeat dosing in female monkeys (n = 6/group). AMG 517 caused an increase of approximately 1.1ºC on day 1 at 3 hrs post administration but not on days 8 or 28. Asterisks indicate p < 0.05 compared with vehicle-treated controls (ANOVA with Dunnett’s post-hoc test).

Figure 6. (A) Antipyretic, acetaminophen suppresses or masks the TRPV1 antagonist-induced hyperthermia in rats. At 30 min, either vehicle (black, red) or 3 mg/kg AMG8163 (blue, green) was administered, and at 150 min, either vehicle (black, blue) or 300 mg/kg acetaminophen (blue, red) was administered. Acetaminophen decreases body temperature when administered alone and reverses AMG8163-induced increase in the body temperature to the vehicle control level. The shaded boxes indicate stress-induced increase in body core temperature. (B) Acetaminophen decreases body temperature when administered alone and suppresses AMG8163-induced increase in the body temperature to the vehicle control level. Each bar represents average ± SD of body temperature between 180-240 min in (A).
Table 1. Effects of AMG 517 on exogenous and proposed endogenous chemical ligands and heat activation of CHO cells recombinantly expressing TRPV1 in agonist-induced $^{45}$Ca$^{2+}$ calcium uptake assays. Since the proposed endogenous ligands are weak agonists, they were used to activate TRPV1 at sensitizing conditions (pH 6). IC$_{50}$ values shown are in nanomolar (average ± SD, n = 3-12). ND indicates not determined.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>Rhesus Monkey</th>
<th>Cynomolgus Monkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin (0.5 µM)</td>
<td>0.76 ± 0.4</td>
<td>1.01 ± 0.7</td>
<td>1.9 ± 0.8</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Proton (pH 5)</td>
<td>0.62 ± 0.27</td>
<td>0.5 ± 0.23</td>
<td>0.63 ± 0.3</td>
<td>0.47 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Heat (45°C)</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.7</td>
<td>ND</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>NADA (7 µM)</td>
<td>19.9 ± 10.8</td>
<td>9.1 ± 4.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Anandamide (10 µM)</td>
<td>3.2 ± 1.4</td>
<td>1.1 ± 0.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>OLDA (7 µM)</td>
<td>7.9 ± 4.2</td>
<td>4.2 ± 2.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HPETE (50 µM)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LB$_4$ (100 µM)</td>
<td>0.5 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 1

A

AMG 517

\[ \text{F}_3\text{C} - \begin{array}{c}
\text{O} \\
\text{NHAc}
\end{array} \]

B

\begin{align*}
\text{AMG 517 (nM)} & \\
\text{\% max } 45\text{Ca}^{2+} \text{ uptake} & \\
-10 & -9 & -8 & -7 & -6 & -5 & -4
\end{align*}

<table>
<thead>
<tr>
<th>Capsaicin, log [M]</th>
<th>0</th>
<th>4</th>
<th>12</th>
<th>37</th>
<th>111</th>
<th>333</th>
</tr>
</thead>
<tbody>
<tr>
<td>% max 45Ca^{2+} uptake</td>
<td>0</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td>100</td>
<td>125</td>
</tr>
</tbody>
</table>

D

\begin{align*}
\text{AMG 517 (nM)} & \\
\text{\% max } 45\text{Ca}^{2+} \text{ uptake} & \\
-10 & -9 & -8 & -7 & -6 & -5 & -4
\end{align*}

| Capsaicin, log [M] | 0 | 4.1 | 12.3 | 37.0 | 111.1 | 333.3 | 1000 |
|--------------------|---|-----|------|-------|--------|--------|
| % max 45Ca^{2+} uptake | 0 | 25   | 50   | 75    | 100    | 125    |

C

\begin{align*}
\log (\text{DR-1}) & \\
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9
\end{align*}

\[ K_b \text{ 4.2 nM} \]

E

\begin{align*}
\log (\text{DR-1}) & \\
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9
\end{align*}

\[ K_b \text{ 6.2 nM} \]
Figure 2

A 2-APB activation - rat TRPV2

B 2-APB activation - human TRPV3

C 4α-PDD activation - human TRPV4

D AITC activation - human TRPA1

E Icilin activation - human TRPM8
Figure 4

**A**

- Post Drug Latency
- Plasma levels

**B**

- Post Drug Latency
- Plasma Levels

**C**

- AMG 517 (0.3 mg/kg)
- AMG 517 (1 mg/kg)
- AMG 517 (3 mg/kg)
- Vehicle

**D**

- 30 mg/kg AMG 517 (n = 3)
- 10 mg/kg AMG 517 (n = 3)
- Vehicle (n = 6)

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5

A

Temperature (°C)

Vehicle (n=7)  AMG8163 (n=6)

lights on  lights off

Time (hour)

0 6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96

B

Temperature (°C)

vehicle  AMG8163

Day

1 2 3 4

Day 1  Day 8  Day 28

C

Temperature (°C)

Vehicle (n = 6)  30 mg/kg/day AMG 517 (n = 6)  500 mg/kg/day AMG 517 (n = 6)

Day

PreDose  3 hours  24 hours

Day 1  Day 8  Day 28
Figure 6

A

B

This article has not been copyedited and formatted. The final version may differ from this version.

Experimental temperatures over time for different treatment groups. The data show a significant difference (*) in temperature between groups at different time points.