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Antinociceptive Profile of ARN19702, (2-Ethylsulfonylphenyl)-[(2S)-4-(6-fluoro-1,3-benzothiazol-2-yl)-2-methylpiperazin-1-yl] methanone, a Novel Orally Active *N*-Acylethanolamine Acid Amidase Inhibitor, in Animal Models

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ABSTRACT

N-Acylethanolamine acid amidase (NAAA) is an N-terminal cysteine hydrolase that stops the physiologic actions of palmitoylethanolamide, an endogenous lipid messenger that activates the transcription factor, peroxisome proliferator–activated receptor-α. We have previously reported that the compound ARN19702 [(2-ethylsulfonylphenyl)-[(2S)-4-(6-fluoro-1,3-benzothiazol-2-yl)-2-methylpiperazin-1-yl]methanone] is an orally active, reversible NAAA inhibitor (IC_{50} on human NAAA = 230 nM) that produces remarkable protective effects against multiple sclerosis in mice. In the present study, we assessed the profile of ARN19702 in mouse and rat models of acute and neuropathic pain. Oral administration in male mice attenuated in a dose-dependent manner the spontaneous nocifensive response elicited by intraplantar formalin injection and the hyper-sensitivity caused by intraplantar carrageenan injection, paw incision, or sciatic nerve ligation. In male rats, ARN19702 reduced nociception associated with paclitaxel-induced neuropathy without development of subacute antinociceptive tolerance. Finally, ARN19702 (30 mg/kg, oral) did not produce place preference or alter exploratory motor behavior in male mice. The findings support the conclusion that NAAA is a suitable molecular target for the discovery of efficacious analgesic drugs devoid of rewarding potential.

SIGNIFICANCE STATEMENT

This study evaluated the pharmacological profile of the orally bioavailable *N*-acylethanolamine acid amidase (NAAA) inhibitor (2-ethylsulfonylphenyl)-[(2S)-4-(6-fluoro-1,3-benzothiazol-2-yl)-2-methylpiperazin-1-yl]methanone (ARN19702) in mouse and rat models of neurogenic and inflammatory pain. The compound's potential rewarding and sedative effects were also examined. It is concluded that ARN19702 exhibits a broad analgesic profile that can be generalized across rodent species. The findings point to NAAA as a control node in the processing of neuropathic and inflammatory pain and to ARN19702 as a lead to uncover novel pain therapeutics devoid of addictive potential .

Introduction

Fatty acid ethanolamides (FAEs), including palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), are a family of bioactive lipid messengers that regulate several physiologic and pathologic processes, including nociception (Piomelli and Sasso 2014; Artukoglu et al., 2017; Suardíaz et al., 2007), inflammation (Impellizzeri et al., 2015), and energy balance (Piomelli, 2013). They are produced in mammalian cells by the action of the zinc-containing hydrolase *N*-acylphosphatidylethanolamine phospholipase D (Okamoto et al., 2004) and exert their biologic actions by engaging the nuclear transcription factor peroxisome proliferator–activated receptor-α (PPAR-α) (Fu et al., 2005; Lo Verme et al., 2005).

The FAEs are deactivated via enzymatic hydrolysis mediated primarily by two structurally and functionally distinct lipid amidases: *N*-acylethanolamine acid amidase (NAAA) and fatty acid amide hydrolase (Piomelli et al., 2020). NAAA is an N-terminal nucleophile cysteine hydrolase that strongly prefers PEA over other FAEs (Tsuboi et al., 2005; Scalvini et al., 2020). Fatty acid amide hydrolase, instead, belongs to the serine amidase family of enzymes and preferentially hydrolyzes the endocannabinoid anandamide (Desarnaud et al., 1995; Cravatt et al., 1996) along with other lipid amides (Lodola et al., 2015).

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D.P. is an inventor in patent applications owned by the University of California, Irvine, which describe *N*-acylethanolamine acid amidase inhibitors. Y.F., O.S., and R.C. have no conflict of interest.
[1]Y.F. and O.S. contributed equally to this work.
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ABBREVIATIONS: **ABBREVIATIONS:** ARN077, 5-phenylpentyl *N*-[(2S,3R)-2-methyl-4-oxo-oxetan-3-yl] carbamate; ARN19702, (2-ethylsulfonylphenyl)-[(2S)-4-(6-fluoro-1,3-benzothiazol-2-yl)-2-methylpiperazin-1-yl]methanone; CNS, central nervous system; CPP, conditioned place preference; FAE, fatty acid ethanolamide; NAAA, *N*-acylethanolamine acid amidase; PEA, palmitoylethanolamide; PPAR-α, peroxisome proliferator–activated receptor-α.

Since its molecular cloning by Natsuo Ueda and colleagues (Ueda et al., 1999) and the invention of its first selective inhibitors (Solorzano et al., 2009), NAAA has emerged as a molecular target for the treatment of pain, inflammation, and addictive disorders (Sagheddu et al., 2019; Piomelli et al., 2020; Fotio et al., 2021). Indeed, our group and others have discovered several classes of small-molecule NAAA inhibitors to illuminate the role played by NAAA in pain control (Piomelli et al., 2020). The first such compound, β-lactone *N*-[(3S)-2-oxo-3-oxetanyl]-3-phenylpropanamide, inhibited rat NAAA with submicromolar potency (IC_{50} = 0.42 μM). Its local administration elevates PEA levels in activated immune cells and blocks expression of apoptosis markers caused by spinal cord injury (Solorzano et al., 2009). A more potent and selective derivative of *N*-[(3S)-2-oxo-3-oxetanyl]-3-phenylpropanamide, ARN077 (5-phenylpentyl *N*-[(2S,3R)-2-methyl-4-oxo-oxetan-3-yl] carbamate), inhibits human NAAA with single-digit nanomolar potency (IC_{50} = 7 nM) and attenuates pain-related responses when applied topically in mouse and rat models (Khasabova et al., 2012; Sasso et al., 2013). Despite its usefulness as a topical agent, ARN077 lacks metabolic stability and cannot be administered systemically, a limitation that was overcome by the development of more stable families of inhibitors which act through either covalent or noncovalent mechanisms (Piomelli et al., 2020). The latter include the benzothiazole-piperazine derivative ARN19702 (2-ethylsulfonylphenyl)-[(2S)-4-(6-fluoro-1,3-benzothiazol-2-yl)-2-methylpiperazin-1-yl]methanone), which is relatively potent (IC_{50} for human NAAA = 230 nM), selective for NAAA, and active after oral administration (Migliore et al., 2016). ARN19702 enters the central nervous system (CNS), albeit incompletely (plasma/brain ratio = 0.2), and alleviates symptoms of multiple sclerosis in mice (Migliore et al., 2016).

Here, we evaluated the pharmacological profile of orally administered ARN19702 in mouse and rat models of neurogenic and inflammatory pain. In male mice, the compound attenuated the spontaneous nocifensive response to intraplantar formalin injection as well as hyperalgesia and allodynia caused by intraplantar carrageenan injection, paw incision, or chronic ligation of the sciatic nerve. In male rats, ARN19702 reduced nociception associated with paclitaxel-induced neuropathy. Despite its significant antinociceptive activity, the compound did not produce place preference or alter exploratory motor behavior in male mice.

Materials and Methods

Animals. We used male CD1 mice (20–25 g, Charles River, Wilmington, MA) and Sprague-Dawley rats (200–220 g, Charles River, Calco, Italy). The animals were maintained in a pathogen-free environment on a 12-hour light/dark cycle at controlled temperature (22°C) and humidity (55%–60%). Food and water were available ad libitum. They were allowed to familiarize themselves to their housing conditions for at least 7 days upon arrival and were randomly assigned to treatment groups. Before the start of the experiments, the animals were handled for 3 consecutive days (~3 minutes per animal/day), and testing was conducted during the light phase of the light/dark cycle. Efforts were made to minimize the number of animals used and their discomfort. The study complied with the National Institutes of Health Guide of the Care and Use of Laboratory Animals and the recommendation of the International Association for the Study of Pain. Experimental procedures were approved by the Animal Care and Use Committees of the University of California, Irvine and the University of Camerino (Italy).

Chemicals. ARN19702 was synthesized and purified following an established protocol (Migliore et al., 2016; Piomelli et al., 2020). λ-Carrageenan, formalin, paclitaxel, morphine hydrochloride, and gabapentin were purchased from Sigma-Aldrich (St. Louis, MO). All solvent and chemicals were of the highest available grade.

Drug Administration. Drug solutions were prepared shortly before use. ARN19702 was dissolved in polyethylene glycol 400/Tween-80/distilled water (15/15/70, vol) and administered orally 1 hour before behavioral measurements. Paclitaxel was dissolved in Cremophor EL/ethanol/distilled water (10/10/80, vol) and administered by intraperitoneal injection. Gabapentin was dissolved in distilled water and injected intraperitoneally 30 minutes before testing. Morphine hydrochloride was dissolved in distilled water and injected subcutaneously immediately before testing. Drugs and vehicles were administered in a volume of 1 ml/kg to rats and 10 ml/kg to mice.

Animal Models

Carrageenan-Induced Hypersensitivity. λ-Carrageenan was dissolved in distilled water to obtain a 1% solution (weight/vol) and was sonicated for a few seconds. 20 μl of this solution were injected into the plantar surface of the right hind paw of lightly restrained adult male CD1 mice (Morris, 2003).

Formalin-Induced Spontaneous Pain. We injected formalin (1% vol, 20 μl) or saline into the plantar surface of the right hind paw of mice. After injection, the animals were immediately transferred to a transparent observation chamber where nocifensive behavior (time spent licking or biting the injected paw, number of paw shakings) was videorecorded for 60 minutes and subsequently quantified by a blinded observer.

Paw Incision–Induced Hypersensitivity. Paw incisions were performed as described (Brennan, 1999). Briefly, mice were anesthetized by inhalation of 2%–3% isoflurane in O_2. Under aseptic conditions, a longitudinal incision (>10 mm) was made through the skin and fascia of the plantar surface of the hind paw. The plantaris muscle was elevated and incised longitudinally. After hemostasis with gentle pressure, the skin was sutured with a 4–0 nylon on a FS-2 needle (Ethicon, USA). After surgery, the mice were allowed to recover from anesthesia and then transferred to clear plexiglass chambers, where they were allowed to familiarize themselves to the environment for 30 minutes before starting the behavioral test.

Sciatic Nerve Ligation. The sciatic nerve was ligated as described (Bennett and Xie, 1988; Bennett et al., 2003). Briefly, the mice were anesthetized by inhalation of 2%–3% isoflurane in O_2, and the right common sciatic nerve was exposed at the level of the middle thigh by blunt dissection under aseptic conditions. Proximal to the trifurcation, the nerve was cleaned from surrounding connective tissue, and 3 chromic cat gut ligatures (4–0, Ethicon, Somerville) were loosely tied around it at 1-mm intervals. The wound was closed with a single muscle suture and skin clips. In sham-operated animals, the nerve was exposed but not tied. Operated mice were returned to their home cages for recovery. Test compounds or vehicle were given by the oral or intraperitoneal route on days 3, 7, and 14 after surgery.

Paclitaxel-Induced Painful Neuropathy. Paclitaxel neuropathy was induced as described (Polomano and Bennett, 2001; Polomano et al., 2001). Sprague-Dawley rats were randomly divided into four groups (n = 8 per group) and given intraperitoneal injections of paclitaxel (1 mg/kg) or its vehicle (10% Cremophor EL/10% ethanol/80% distilled water) on four alternate days (1, 3, 5, and 7). The cumulative dose of paclitaxel was 4 mg/kg. Oral administration of ARN19702 (3 and 10 mg/kg) began 24 hours after the last paclitaxel injection (acute) and continued for 7 consecutive days (subchronic). Mechanical allodynia was measured 1 hour after drug treatment.

Behavioral Measurements. Mechanical allodynia was measured using a dynamic plantar aesthesiometer (Ugo Basile). Individual animals were randomly placed in transparent plexiglass chambers with a

mesh floor and allowed to acclimatize for 45 minutes. After this habituation period, an automated stainless steel filament was used to apply to the plantar surface of the injured paw a gradually increasing mechanical pressure ranging from 0 to 5 g over 10 seconds for mice and 0 to 50 g over 20 seconds for rats. Three forces at which animals withdraw their paw were measured and averaged to determine the paw withdrawal threshold (in grams). Thermal hyperalgesia was assessed using a Hargreaves plantar test apparatus (San Diego Instruments, CA). Mice were individually placed in transparent plexiglass enclosures with a glass floor. After a 45-minute habituation period, the plantar surface of the hind paws was exposed to a beam of radiant heat through the glass floor. The cut-off time was set at 15 seconds. The latency to remove the paw from the radiant heat (paw withdrawal latency, in seconds) was recorded automatically. Each paw was tested three times with a 2-minute interval between stimuli, and the mean paw withdrawal threshold and latency were calculated. Paw edema was measured with a plethysmometer (Ugo Basile) and expressed as the difference (Δ paw volume, in ml) between the ipsilateral paw thickness and the contralateral counterpart. Motor activity was recorded for 10 minutes in a novel environment using an automated system (TSE, Bad Homburg, Germany) consisting of 24 cages equipped with an X-Y matrix of infrared sensors. Each time an infrared light was interrupted, the computer recorded the number of beams broken.

Conditioned Place Preference. The CPP apparatus consisted of two contiguous acrylic chambers (26 × 30 × 40 cm) connected by a 10-cm wide doorway that can be opened by removing the guillotine-style door. The two compartments are distinct based on tactile and visual traits. The right chamber of the apparatus is made of black and white striped walls, and a removable mesh floor. The left chamber comprised uniform gray walls and smooth flooring. The test consisted of three phases: habituation, conditioning, and preference assessment.

Pretest: On day 1, the mice received vehicle intraperitoneally and were placed at the center of the apparatus with the door open. They were allowed to freely explore both chambers for 15 minutes and were then returned into their home cage. Individual mice that showed preference for one chamber were excluded from the analyses.

Conditioning: On days 2, 4, 6, and 8, mice received ARN19702 (30 mg/kg, i.p.), morphine (10 mg/kg, s.c.), or their vehicles and were immediately placed into the appropriate chamber (drug-paired chamber) of the CPP box for 15 minutes. On days 3, 5, 7, and 9, the mice were administered vehicle and were immediately placed in the opposite chamber (vehicle-paired chamber) for 15 minutes. The choice of drug associated with each chamber and preferences for drug or vehicle sides were matched across animals based on exploratory behavior during habituation.

Preference test: 24 hours after the conditioning session, mice were injected with vehicle (i.p.) and placed at the center of the apparatus with the door open. They were allowed to freely explore both chambers for 15 minutes and were returned into their home cage. Time (s) spent in each chamber was evaluated to determine preference.

Statistical Analyses. Results are expressed as the mean ± S.D. The effects of ARN19702 on carrageenan, formalin, and postoperative (time-course) pain were analyzed by two-way repeated measure ANOVA with treatment as the between factor and time as the within factor. The effects of ARN19702 on sciatic nerve ligation, paclitaxel-induced neuropathic pain, and CPP were analyzed by one-way ANOVA with treatment as the between factor. The effects of ARN19702 on locomotor activity were analyzed by unpaired two-tailed Student's t test. Areas under the curve were calculated using the trapezoidal rule. When appropriate, Dunnett's or Bonferroni's post hoc analyses were applied. *$p < 0.05$ versus vehicle, **$p < 0.01$ versus vehicle, ***$p < 0.001$ versus vehicle. Analyses were conducted using Prism software (GraphPad Software, San Diego, CA, Version 8.4.2).

Results

Effects of ARN19702 on Carrageenan-Induced Edema and Hyperalgesia. We injected carrageenan (1% weight/vol) into the hind paw of five groups of male mice ($n = 6$ per group) 1 hour after oral administration of ascending doses of ARN19702 (in mg/kg: 0.1, 0.3, 1, and 3) or its vehicle. Carrageenan produced an increase in paw volume of vehicle-treated mice, which was attenuated by ARN19702 in a dose- and time-dependent manner (Fig. 1A). Withdrawal latency to radiant heat was reduced by ~70% in inflamed paws of vehicle-treated mice. This effect was also dose- and time-dependently alleviated by ARN19702 (Fig. 1B).

Effects of ARN19702 on Formalin-Evoked Spontaneous Pain. Next, we assessed the ability of ascending doses of ARN19702 (in mg/kg: 0.1, 0.3, 1, and 3) to prevent formalin-induced spontaneous pain behavior in male mice. As expected, formalin evoked an immediate nocifensive reaction (paw licking/biting and shaking) consisting of two temporally distinct phases (Fig. 2, A–C; early phase: 0–15 minutes and late phase: 20–60 minutes). The response was markedly attenuated, in a dose- and time-dependent manner, by a single oral administration of ARN19702 1 hour before formalin (Fig. 2, A-C). At its highest dose (3 mg/kg), ARN19702 attenuated early and late phase of the response by ~76% ($P < 0.0001$) and ~82% ($P < 0.0001$), respectively.

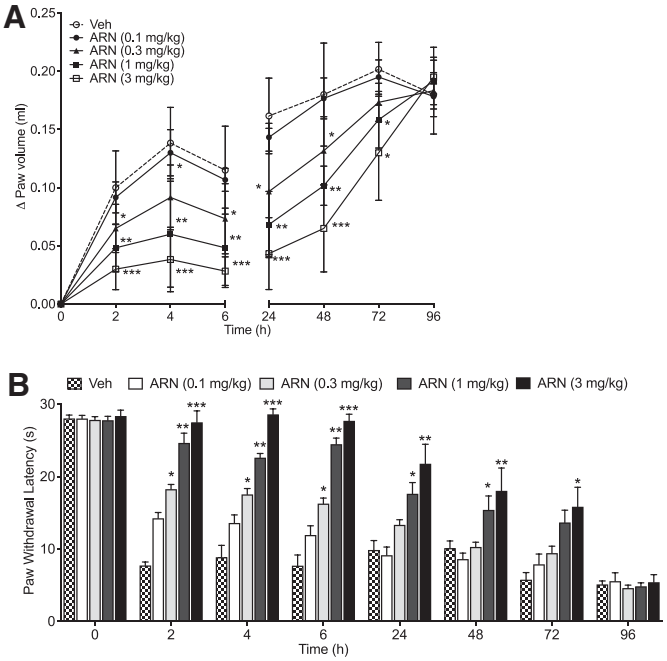

Fig. 1. Effects of the orally active NAAA inhibitor ARN19702 on carrageenan-induced inflammatory pain in mice. Mice received intraplantar injection of carrageenan (5%, vol/vol) 1 hour after oral administration of ARN19702 [ARN (0.1; 0.3; 1; 3 mg/kg)] or its vehicle (Veh). Paw edema (Δ paw volume; injected paw thickness minus noninjected, in ml) and heat hyperalgesia [paw withdrawal latency (s)] were measured for the following 96 hours (h). Time-course of the effect of ARN19702 on (A) paw edema and (B) paw withdrawal latency. Data are expressed as mean ± S.D ($n = 10$ per group) and were analyzed by two-way repeated measures ANOVA followed by Bonferroni's post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from vehicle-treated animals.

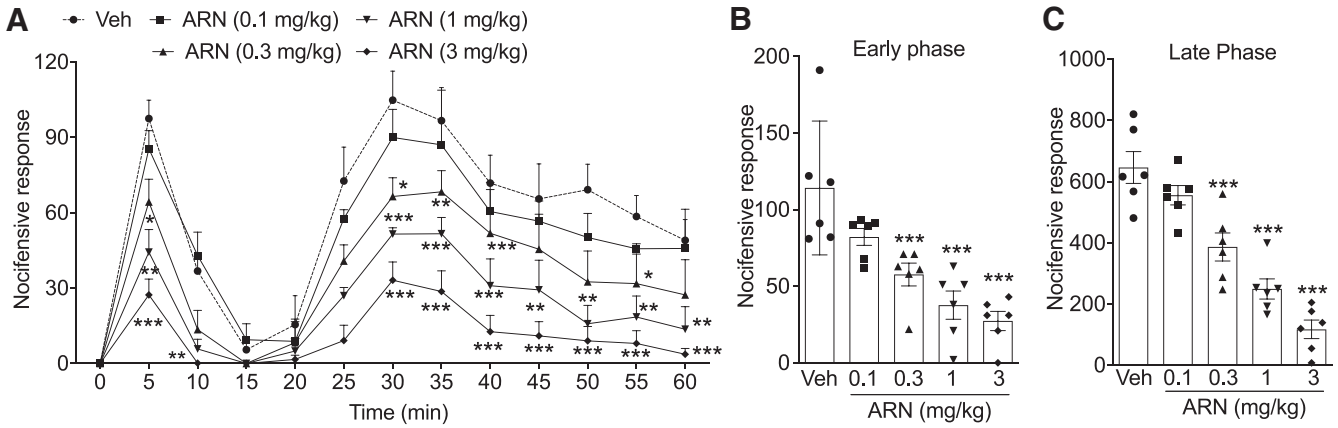

Fig. 2. Effects of ARN19702 on formalin-induced spontaneous pain in mice. Mice received intraplantar formalin (1%, vol/vol) injections 1 hour after oral administration of ARN19702 [ARN (0.1; 0.3; 1; 3 mg/kg)] or its vehicle (Veh). (A) Time-course of the acute nocifensive response to formalin. (B and C) Cumulative scores of the early [(0–15 minute), B] and late phase [(20–60 minute), C] of the response. Data are expressed as mean ± S.D (n = 6 per group) and were analyzed by two-way repeated measures (A) or one-way (B and C) ANOVA followed by Bonferroni's and Dunnett's post hoc test, respectively. Min: minutes. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus vehicle controls.

Effects of ARN19702 on Postoperative Hyperalgesia and Mechanical Allodynia. We made longitudinal incisions on the hind paws of five groups (n = 10 per group) of anesthetized mice and assessed sensory responses after they had fully recovered (see *Materials and Methods*). As illustrated in Fig. 3, the incised paws of vehicle-treated animals exhibited decreased withdrawal latency (Fig. 3A) and threshold (Fig. 3B) (~83%, $P < 0.0001$ and ~58%, $P < 0.0001$, respectively). These sensory abnormalities were alleviated in a dose- and time-dependent manner by a single oral administration of ARN19702 (in mg/kg: 1, 3, 10, and 30). Two hours after treatment, the highest dose of ARN19702 (30 mg/kg) reduced heat hyperalgesia and mechanical allodynia by 91%–93% ($P < 0.0001$), an effect that tapered off 72 hours later (Fig. 3, A and B).

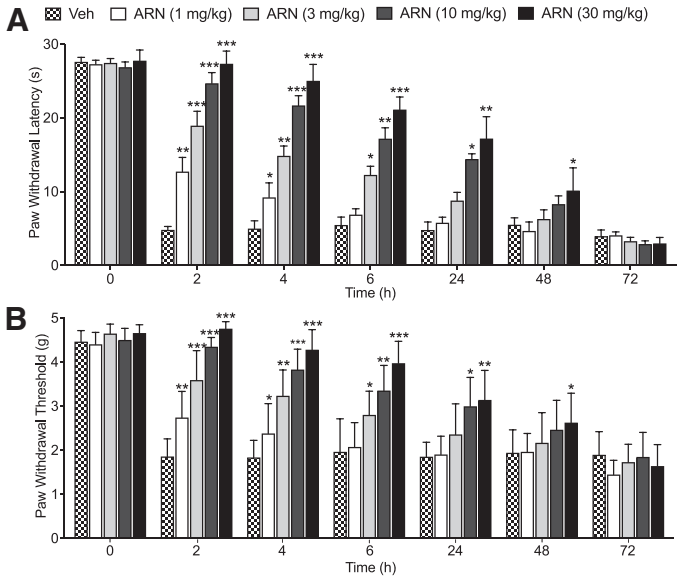

Fig. 3. Effects of ARN19702 on postoperative sensory abnormalities in mice. ARN19702 [ARN (1, 3, 10, 30 mg/kg)] or its vehicle (Veh) were administered orally 1 hour (h) after paw incision surgery. Heat hyperalgesia (A) and mechanical allodynia (B) were measured at various time points after surgery. Data are expressed as mean ± S.D (n = 10 per group) and were analyzed by two-way ANOVA followed by Dunnett's post hoc test. s, second; g, gram. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from vehicle-treated animals.

Effects of NAAA Inhibition on CCI -Induced Hyperalgesia and Mechanical Allodynia. We asked whether ARN19702 might alleviate established hyperalgesia and allodynia caused by sciatic nerve ligation in male mice. We administered ARN19702 (in mg/kg: 1, 3, 10, and 30) orally on days 3, 7, and 14 after the surgery and measured sensory abnormalities in the following hours after each dosing (see *Materials and Methods*). In vehicle-treated mice, withdrawal latency (Fig. 4, A, B, C, and E) and threshold (Fig. 4, D and F) in injured paws were lowered by ~86% ($P < 0.0001$) throughout the experimental period compared with the sham-operated group. Statistical analysis of the data revealed that a single administration of ARN19702 was sufficient to attenuate heat hyperalgesia (Fig. 4, A, B, C, and E) and mechanical allodynia (Fig. 4, D and F) in a dose- and time-dependent manner ($P < 0.0001$). These effects were compared, in a separate group of mice, to those of gabapentin (50 mg/kg, oral), an antiepileptic agent used as first-class treatment of neuropathic pain (Wiffen et al., 2017; Moore et al., 2018). As expected from our previous work (Sasso et al., 2013; Fotio et al., 2019), gabapentin attenuated ($P < 0.001$) heat hyperalgesia (Fig. 4, C and E) and mechanical allodynia (Fig. 4, D and F) by ~29%.

Effect of NAAA Inhibition on Paclitaxel-Induced Peripheral Neuropathy. Next, we examined whether ARN19702 alleviates mechanical allodynia associated with chemotherapy-induced neuropathy. Sprague-Dawley rats (n = 8 per group) were given the antineoplastic drug paclitaxel (cumulatively 4 mg/kg, i.p.) and were then randomly assigned to treatment groups. ARN19702 (3 and 10 mg/kg) was orally administered 1 hour after the last paclitaxel injection. As illustrated in Fig. 5A, a single dose of the NAAA inhibitor decreased established mechanical allodynia by 30% (3 mg/kg) and 52% (10 mg/kg) ($P < 0.0001$). In a separate experiment, ARN19702 was administered orally (3 and 10 mg/kg) to neuropathic rats once daily for 7 consecutive days. The treatment reversed mechanical allodynia (Fig. 5B), suggesting that there was no tolerance to the antinociceptive effects of ARN19702.

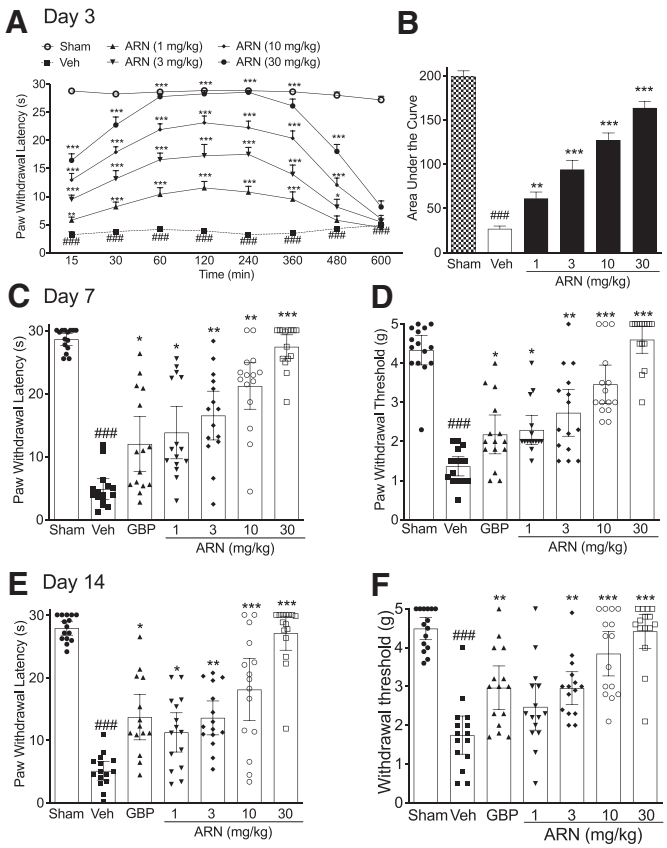

Fig. 4. Effects of ARN19702 (ARN) on CCI-evoked heat hyperalgesia and mechanical allodynia in mice. ARN19702 (1, 3, 10, and 30 mg/kg) was administered orally to neuropathic mice on days 3 (A and B), 7 (C and D), and 14 (E and F) after CCI surgery. Behavioral testing was conducted at various time points after treatment. (A and B) Time-course of the effects of ARN19702 on heat hyperalgesia on day 3 after surgery. Dose-response curves of the effects of ARN19702 on CCI-induced heat hyperalgesia (C and E) and mechanical allodynia (D and F) measured 2 hours after treatment on postsurgical days 7 and 14. Data are expressed as mean ± S.D (n = 18 per group) and were analyzed by two-way (A) or one-way (B–F) ANOVA followed by Dunnett's or Bonferroni's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle controls. $^{\#\#\#}P < 0.001$ versus sham. GBP, gabapentin; veh, vehicle; s, second; g, gram; CCI, Chronic Constriction Injury.

Effect of NAAA Inhibition on Conditioned Place Preference and Motor Behavior. Finally, we assessed whether ARN19702 might produce place preference or alter motor activity. ARN19702 (ARN-Veh; 30 mg/kg, oral), morphine (Mor-Veh; 10 mg/kg; s.c.) or their respective vehicles (Veh-Veh) were administered to male CD1 mice (n = 10 per group) and tested as conditioning drugs (see *Materials and Methods*). Statistical analysis of the data demonstrated that administration of ARN19702 caused neither place preference (Fig. 6A) nor changes in exploratory motor activity, as shown by both the number of crossing events at the central doorway (Fig. 6B) and the number of infrared beams broken (Fig. 6C). The place conditioning effects of ARN19702 were compared in a separate group of mice (n = 10) to those of morphine (Mor, 10 mg/kg, s.c.), a first-class treatment of moderate to severe pain (https://www.ncbi.nlm.nih.gov/books/NBK526115). As anticipated, morphine produced a ~22-fold increase ($P < 0.001$) in time spent in the drug-paired chamber (Fig. 6A).

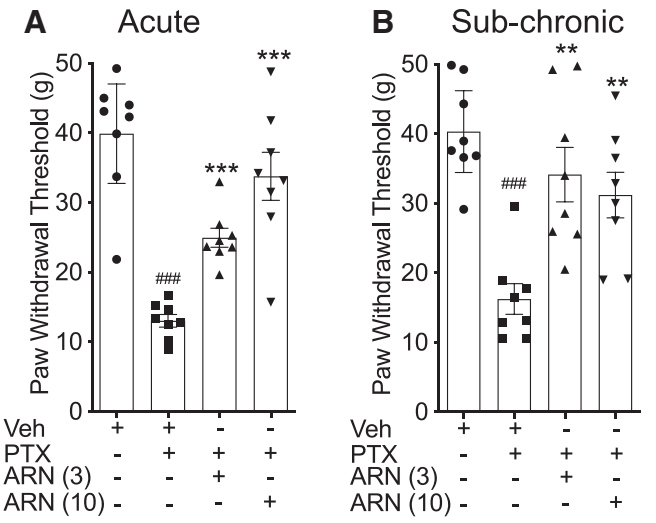

Fig. 5. Effects of ARN19702 on paclitaxel-induced painful neuropathy in rats. ARN19702 [ARN (3 and 10 mg/kg)] or its vehicle (Veh) were orally administered to rats 1 hour after injection of paclitaxel (PTX) on day 7. (A) Effects of acute oral administration of ARN19702 on PTX-induced mechanical allodynia on day 7. (B) Effects of subchronic oral administration of ARN19702 on PTX-induced mechanical allodynia on day 14. Data are expressed as mean ± S.D (n = 8 per group) and were analyzed by one-way ANOVA followed by Dunnett's post hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$ versus PTX-treated groups. $^{\#\#\#}P < 0.001$ versus vehicle controls. g, gram.

Discussion

More than 100 million American adults are affected by pain, and its management represents a scientific and medical challenge (Zelaya et al., 2020). In the present study, we show that oral administration of the novel brain-penetrant NAAA inhibitor, ARN19702 (Migliore et al., 2016), dose-dependently alleviated, in male mice, the spontaneous nocifensive response to formalin as well as hyperalgesia and allodynia caused by carrageenan, paw incision, or sciatic nerve ligation. Furthermore, in male rats, ARN19702 reduced nociception associated with paclitaxel-induced peripheral neuropathy without development of subacute tolerance to its antinociceptive effects. Of note, the inhibitor was as effective as the first line analgesic gabapentin in attenuating hyperalgesia and allodynia evoked by nerve damage. A plausible interpretation of these findings is that ARN19702 reduces nociceptive responses in mice and rats by blocking NAAA-catalyzed hydrolysis of PEA, an endogenous PPAR-α ligand whose antinociceptive and anti-inflammatory actions are well characterized (Piomelli and Sasso, 2014; Impellizzeri et al., 2015; Artukoglu et al., 2017). Interestingly, we observed that the antinociceptive effects of ARN19702 lasted longer (>48 hours) than predicted from its plasmatic half-life (?104 minutes) after oral administration (Migliore et al., 2016). Further experiments are needed to reconcile this discrepancy, but it is reasonable to suggest that it might stem from the mechanism of action of NAAA inhibitors, which activate (through PEA) PPAR-α–dependent transcription of genes involved in the control of nociceptive signaling. This view is supported by the fact that PEA itself exerts long-lasting antinociceptive effects (Mazzari et al., 1996, LoVerme et al., 2006), despite its short half-life in circulation (?12 minutes) (Gabrielsson et al., 2016).

Since alterations in motor activity and compulsive drug seeking are hallmark side effects that hamper the clinical use

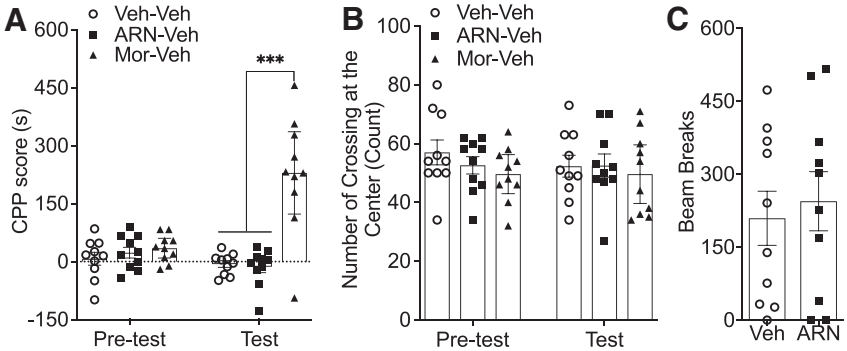

Fig. 6. Effect of ARN19702 on place conditioning and motor activity. (A and B), ARN19702 (ARN-Veh; 30 mg/kg, oral), morphine (Mor-Veh; 10 mg/kg; s.c.), or their respective vehicles (Veh-Veh) were administered to male CD1 mice and tested as conditioning drugs (see *Materials and Methods* section). (A) CPP score before (pretest) and after (test) conditioning experiments. The values (CPP score) represent the difference in time (in seconds) (s) spent in the drug-paired versus the vehicle-paired compartment. (B) Number of crossing at the central doorway. (C) Another group of mice were treated with ARN19702 (ARN; 30 mg/kg, oral) or its vehicle (Veh), and motor activity was measured and reported as "beam breaks" during a 10-minute trial in a novel environment. Data are shown as mean ± S.D (n = 10 per group) and were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. ***$P < 0.001$ versus morphine-treated groups.

of potent analgesic drugs such as the opioids (Robinson and Berridge, 1993; Zernig et al., 2007), we tested ARN19702 as a conditioning drug and found that the inhibitor failed to produce place preference. These results suggest that ARN19702 is devoid of rewarding properties.

Evidence points to an important role for NAAA in the control of nociceptive and inflammation responses. Tissue deficits in NAAA's primary substrate, PEA, (Tsuboi et al., 2005; Scalvini et al., 2020) have been documented in painful and inflammatory disorders (Richardson et al., 2008; Suarez et al., 2012). Similarly, experiments in animal models suggest that organ damage and proinflammatory stimuli decrease PEA signaling (Solorzano et al., 2009; Borrelli et al., 2015; Bonezzi et al., 2016). Although human inflammatory states are only partially captured by animal models, it is reasonable to hypothesize that endogenous PEA might be involved in a gaiting mechanism that prevents peripheral nociceptive information to access the CNS (Piomelli and Sasso, 2014; Piomelli et al., 2014). If this is the case, pharmacological tools aimed at increasing the biologic actions of PEA might lead to the discovery of novel therapeutic strategies for the management of acute and chronic pain. Indeed, preclinical and clinical studies have documented PEA's ability to significantly alleviate nociceptive and inflammatory responses by engaging PPAR-α (Lo Verme et al., 2005; LoVerme et al., 2006; Solorzano et al., 2009; Gabrielsson et al., 2016; Artukoglu et al., 2017; Piomelli et al., 2020). Unfortunately, the low bioavailability, rapid degradation, and insufficient CNS penetration of PEA limit its efficacy as an oral analgesic agent. An alternative is to magnify PEA signaling by protecting this lipid amide from NAAA-mediated degradation. Supporting this approach, the present results demonstrate that ARN19702 exerts antinociceptive effects in several mechanistically different rodent pain models. Moreover, other studies have documented similar effects with structurally distinct NAAA inhibitors, such as the oxazolidinone imides F215 and F96 (Zhou et al., 2019), the isothiocyanate derivative AM9053 (Alhouayek et al., 2015; Toma et al., 2021), and the β-lactone ARN077 (Sasso et al., 2013). One plausible explanation for these findings is that NAAA inhibitors enhance PEA-dependent modulation of sensory neuron activity (LoVerme et al., 2006; Khasabova et al., 2012), although other mechanisms cannot be excluded.

The present study has several limitations. First, only male mice and rats were used. Since significant differences in pain responses have been reported between male and female animals (Mogil, 2012), studies in both sexes are clearly needed. Second, we did not evaluate changes in PEA levels after treatment with ARN19702. This weakness, however, is mitigated by our prior report showing that this compound effectively inhibits NAAA activity and increases PEA content in the mouse brain (Migliore et al., 2016). Finally, molecular and cellular mechanism(s) engaged by ARN19702 to alleviate nociceptive behavior have not been investigated. Previous work has shown that NAAA inhibitors such as ARN077 act via PEA-mediated recruitment of PPAR-α (Sasso et al., 2013; Piomelli et al., 2020). Nevertheless, accumulated NAAA substrates may also engage other receptors, such as transient receptor potential vanilloid-1 and G protein–coupled receptor 119 (Ahern, 2003; Godlewski et al., 2009). Further experiments are required to shed light on these possibilities.

In conclusion, our results demonstrate that ARN19702 exerts a broad spectrum of antinociceptive effects that is generalized across two rodent species. Importantly, the inhibitor did not produce rewarding-like effects or alteration in motor behavior. Together, the findings confirm the value of NAAA as a molecular target for the treatment of pain and inflammation.

Authorship Contributions

Participated in research design: Fotio, Sasso, Ciccocioppo, Piomelli.
Conducted experiments: Fotio, Sasso.
Performed data analysis: Fotio, Sasso.
Wrote or contributed to the writing of the manuscript: Fotio, Piomelli.

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