Selective Activation of Nociceptor TRPV1 Channel and Reversal of Inflammatory Pain in Mice by a Novel Coumarin Derivative Muralatin L from *Murraya alata*

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Coumarin and its derivatives are fragrant natural compounds isolated from the genus *Murraya* that are flowering plants widely distributed in East Asia, Australia, and the Pacific Islands. *Murraya* plants have been widely used as medicinal herbs for relief of pain, such as headache, rheumatic pain, toothache, and snake bites. However, little is known about their analgesic components and the molecular mechanism underlying pain relief. Here, we report the bioassay-guided fractionation and identification of a novel coumarin derivative, named muralatin L, that can specifically activate the nociceptor transient receptor potential vanilloid 1 (TRPV1) channel and reverse the inflammatory pain in mice through channel desensitization. Muralatin L was identified from the active extract of *Murraya alata* against TRPV1 transiently expressed in HEK-293T cells in fluorescent calcium FlexStation assay. Activation of TRPV1 current by muralatin L and its selectivity were further confirmed by whole-cell patch clamp recordings of TRPV1-expressing HEK-293T cells and dorsal root ganglion neurons isolated from mice. Furthermore, muralatin L could reverse inflammatory pain induced by formalin and acetic acid in mice but not in TRPV1 knock-out mice. Taken together, our findings show that muralatin L specifically activates TRPV1 and reverses inflammatory pain, thus highlighting the potential of coumarin derivatives from *Murraya* plants for pharmaceutical and medicinal applications such as pain therapy.

*Murraya* is a popular genus of flowering plants in the Rutaceae family known for their specific fragrance. Most plants from this genus have been used as traditional Chinese medicines for treating psychogenic pain or somatof orm pain disorders, including toothache, gastralgia, lumbago, rheumatic pain, etc. (1). Previous phytochemical and pharmacological investigations have shown that coumarins isolated from *Murraya* plants are the main bioactive agents responsible for analgesic properties of these medicinal herbs (2). However, the bioactive ingredients have always been a riddle, and their mechanisms of action remain largely unknown.

The transient receptor potential vanilloid member 1 (TRPV1) channel, also known as capsaicin receptor, is a nonselective cation and heat-activated channel with a temperature threshold above 43 °C (3). In addition to chili pepper and temperature, TRPV1 is also activated by acidic pH, and a plethora of other chemicals from plants and toxins (3–7). TRPV1 belongs to the TRPV subfamily that is composed of six members divided into two groups as follows: TRPV1–4 channels that are modestly permeable to Ca2+ and TRPV5–6 channels that are only highly Ca2+-selective, based on their homology and biophysical properties (8, 9). The expression of TRPV1 has been primarily demonstrated in pain pathways, including small diameter primary sensory neurons (10) and keratinocytes in the skin where it plays a key role in nociception induced by capsaicin or noxious thermal stimuli (11, 12). Mice lacking TRPV1 show dramatic reduction of pain hypersensitivity, demonstrating TRPV1 as a potential drug target for inflammatory, neuropathic, and cancer-related pain (13, 14). It has been shown that the capsaicin 8% patch, clinically known as Qutenza, is effective in alleviating neuropathic pain associated with postherpetic neuralgia by reducing TRPV1 expression and decreasing the density of epidermal nerve fibers in the application area (15). Thus, targeting TRPV1 by desensitizing the channel function can serve as an attractive strategy for pain therapy, and screening of natural compounds may lead to discovery of novel and specific modulators for TRPV1 (16).
In this study, we adopted a target-based strategy to screen extracts and individually purified compounds derived from *Murraya* plants against TRP channels. Using a combination of fluorescent calcium assay and electrophysiology as a primary screen and further fractionation of the active extract, we identified a novel coumarin derivative, named muralatin L that can specifically activate TRPV1 and reverse inflammatory pain. Our findings provide a mechanistic explanation for medical use of *Murraya* plants in pain therapy and also a potential for identifying more novel TRPV channel modulators from medicinal herbs.

**Experimental Procedures**

*Isolation of Compound Muralatin L—* The leaves of *Murraya alata* (8.5 kg) were extracted three times with 95% aqueous EtOH (80 liters × 2 h). The extract was evaporated under reduced pressure, and the residual (1.8 kg) was suspended in H$_2$O, and first degreased with petroleum ether, and then partitioned with CHCl$_3$. The CHCl$_3$ extract (fraction A, 500 g) was fractionated by silica gel column chromatography and eluted with a stepwise gradient of petroleum ether/acetone (9:1, 8:2, 7:3, 6:4, and 5:5, v/v) to afford 10 fractions (F1–F10). Fraction (3 g) was further chromatographed over silica gel column chromatography eluting with CH$_2$Cl$_2$ to afford fractions F4a–F4d. F4b (1 g) was further chromatographed by silica gel column chromatography eluting with 7:3, 6:4, and 5:5, v/v) to afford 10 fractions (F1–F10). F4 (3 g) was fractionated by silica gel column chromatography and eluted with a stepwise gradient of petroleum ether/acetone (9:1, 8:2, 7:3, 6:4, and 5:5, v/v) to afford 10 fractions (F1–F10). F4 (3 g) was fractionated by silica gel column chromatography and eluted with a stepwise gradient of petroleum ether/acetone (9:1, 8:2, 7:3, 6:4, and 5:5, v/v) to afford 10 fractions (F1–F10).

*Cell Culture and Transient Transfection of Cells—* HEK-293T cells were plated onto glass coverslips for subculture’s modified Eagle’s medium and 10% fetal bovine serum in 5% CO$_2$. HEK-293T cells were seeded at a density of 30,000 cells/well in FlexStation 3 Microplate Reader (Molecular Devices). HEK-293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with human TRPV1 cDNA. The accession number of TRPV1 cDNA is NM_080704.3. TRPV1 was constructed by primers ATCGAT-GAAGAAATGGAGCAGCA (forward) and ACTGTCACTT-CCTCCCGGAGC (reverse) using LA Taq (TAKARA) and subcloned into Bgl II restriction sites of the pIRES2-EGFP vector. TRPV1-Y511A and TRPV1-Y550A mutants were generated by site-directed mutagenesis using the QuikChange XL kit (Agilent Technologies). 4 µg of individual cDNA were used. All restriction enzymes were purchased either from Invitrogen or Takara, and inserts of all cDNA clones were confirmed by sequencing. Electrophysiological experiments were performed between 18 and 36 h after transfection.

*Intracellular Calcium Measurement by FlexStation 3 Multimode Microplate Reader Assay—* Changes in intracellular calcium level ([Ca$^{2+}$]) in a population of cells were measured by fluorescent calcium-sensitive dyes using the Calcium5 assay kit in FlexStation 3 Microplate Reader (Molecular Devices). HEK-293T cells were seeded at a density of ~30,000 cells/well in 96-well black-walled plates (Thermo) covered with poly-D-lysine. Cells were loaded with dyes from the FLIPR Calcium5 assay kit for 1 h at 37 °C in the presence of 2.5 mM probenecid. Loading and imaging were performed in Hanks’ balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM KH$_2$PO$_4$, 0.1 mM Na$_2$HPO$_4$, 1.3 mM CaCl$_2$, 0.8 mM MgSO$_4$, 5.5 mM glucose, 4 mM NaHCO$_3$, and 20 mM HEPES, pH 7.4). Fluorescence intensity at 525 nm was measured at an interval of 1.6 s, using an excitation wavelength at 485 nm and an emission wavelength at 515 nm (17).

*Calcium Imaging—* HEK-293T cells were loaded with the fluorescent dye 5 µM Fura-2 AM (Beyotime) and 0.02% pluronic for 15 min and washed with a solution containing 145 mM NaCl, 5 mM KCl, 1.25 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM glucose, and 10 mM HEPES. Cells were incubated in the wash buffer for 30 min to allow ester hydrolysis. Ca$^{2+}$ influx was detected by Fura-2 excitation at 340 and 380 nm. 5 µM capsaicin and 500 µM muralatin L were added at the indicated time points. Data were averaged from capsaicin-sensitive cells in the field (18, 19). Cells were imaged under Olympus IX81 microscope. Ca$^{2+}$ influx was observed with MetaFluor software.

**Antinociceptive Tests—** Kunming mice (18–22 g) or C57BL/6 mice were used for experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center and were performed in compliance with national and institutional guidelines for the care and use of laboratory animals.

*Inflammatory Paw of Pain Induced by Formalin—* Inflammatory pain in mouse right hindpaw was induced by intraplantar injection of 0.92% (v/v) formalin dissolved in saline. Paw licking was observed and compared between groups in mice injected intraperitoneally with either 0.2% (v/v) morphine dissolved in saline as positive control or muralatin L (10 or 40 mg·kg$^{-1}$) dissolved in 100 µl of 10% Tween 80-containing saline or just 10% Tween 80-containing saline as vehicle control. Mice were placed individually into open polystyrene cages (20 × 40 × 15 cm). The time spent licking the injected paw was recorded dur-
ing phase I (0–5 min post-injection) and phase II (15–30 min post-injection) (22).

Trpv1 knock-out mice were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences. Nociceptive behavior in mice lacking Trpv1 was induced by intraplantar injection of 1% formalin, capsaicin (1 mM/paw), or muralatin L (10 mg/kg). Muralatin L was dissolved in 100 μl of saline or 10% Tween 80-containing saline. Mice in the control group received the same volume of saline. Injected mice were placed individually into open polyvinyl cages (20 × 40 × 15 cm). Time spent licking the injected paw was observed during the following 40 min.

Abdominal Writhing Induced by Acetic Acid—Mice were injected intraperitoneally with 100 μl of vehicle containing muralatin L (10 or 40 mg/kg) or morphine (10 mg/kg). Muralatin L was dissolved in 100 μl of saline or 10% Tween 80-containing saline. Mice in the control group received the same volume of saline. Injected mice were placed individually into open polyvinyl cages (20 × 40 × 15 cm). Time spent licking the injected paw was observed during the following 40 min.

Abdominal Writhing Induced by Acetic Acid—Mice were injected intraperitoneally with 100 μl of vehicle containing muralatin L (10 or 40 mg/kg) or 0.2% (v/v) morphine dissolved in saline 30 min before intraperitoneal injection of 100 μl of 1.6% (v/v) acetic acid, which induces abdominal contractions and hind limb stretching. The control group received the same volume of saline. Mice were placed into open polyvinyl cages (20 × 40 × 15 cm) immediately after acid challenge, and abdominal contractions were counted cumulatively over a period of 30 min (22).

Thermal Pain Test in Mice—Pre-screening was conducted in mice with a light beam focused on the middle portion of the tail, and mice with withdrawal latency of 4–6 s were selected for subsequent tail-flick test. Test animals were injected intraperitoneally with 100 μl of vehicle containing muralatin L (10 or 40 mg/kg) or morphine 0.2% (v/v) 30 min before heat radiation to the tail. The control group received the same volume of saline. Tail withdrawal latency was measured as the time taken to withdraw the tail the from light beam (22).

Molecular Docking—Molecular docking was carried out using the Maestro software suite (Maestro, version 7.5, Schrödinger, New York). The muralatin L molecule was drawn using the builder tool in Maestro and then optimized for docking in Ligprep. The TRPV1 crystal structure (Protein Data Bank code 3J5R) was prepared for the docking following the Glide standard procedure (23). Grids defining the protein receptor were generated considering the binding mode of capsaicin (24).

Statistical Analysis—All data are expressed as mean ± S.E. Statistical significance was assessed by Student’s t test using Prism 5.0 software. A value of p < 0.05 was considered to represent statistical significance. EC<sub>50</sub> is the concentration for half-maximal effect.

Results

Identification of a Structurally Novel TRPV1 Agonist by Cell-based FlexStation 3 Calcium Assay—The genus Murraya contains nine species in China, and we collected and extracted all these species using 95% aqueous ethanol. A primary screening of these extracts was carried out against TRP channels, including TRPV1, TRPV3, Trpv4, and TRPA1, using FlexStation calcium assay. The extract of M. alata at the concentration of 200 μg/ml (Fig. 1A) was found to be active on TRPV1 but not on the other TRP channels. Thus, this plant was selected for further investigations.

Bioassay-guided fractionation of M. alata showed that fraction A (chloroform extract), which was rich in coumarins from
HPLC and NMR analysis, was the most active portion. Further fractionation of fraction A by extensive separation techniques, including medium pressure liquid chromatography and preparative TLC, identified a novel coumarin, named muralatin L (Fig. 1B). The structure was determined to be 8-[(2S)-2-hydroxy-3-methyl-3-butenyl]-5,6,7-trimethoxycoumarin by comprehensive analysis of data obtained from high resolution mass spectroscopy, infrared spectroscopy, and one- and two-dimensional NMR, and electronic circular dichroism assays (data not shown).

Muralatin L, \( \alpha_{D}^{24} = -37.0 \) (c 0.10, MeOH), was isolated as light yellow oil with a molecular formula of \( \text{C}_{17}\text{H}_{20}\text{O}_{6} \) determined by the high resolution electrospray ionization-mass spectroscopy ion at \( m/z \) 321.1339 [M + H] \(^{+}\) (calculated for \( \text{C}_{17}\text{H}_{21}\text{O}_{6} \), 321.1338). The infrared spectroscopic absorptions indicated the existence of hydroxy \((3468 \text{ cm}^{-1})\) and carbonyl \((1735 \text{ cm}^{-1})\) functionalities. The \(^{1}\text{H}\) and \(^{13}\text{C}\) NMR data (Table 1) were similar to those of omphamurin (25) except for the presence of an additional methoxyl \((\delta_{c} 3.93 \text{ (s), } 6\text{-OCH}_3)\), which was deduced to be located at C-6 by the heteronuclear multiple bond correlations of H-1' and C-7/C-9, H-4 and C-5/C-9/C-10, OCH\(_3\)-5/C-5, OCH\(_3\)-6/C-6, and OCH\(_3\)-7/C-7. The absolute configuration of C-2' was established as S based on the observation of positive sign of band E at 365 nm in its Rh\(_2\) (OCOCF\(_3\))\(_4\)-induced electronic circular dichroism spectrum. Thus, the structure of muralatin L was defined as 8-[(2S)-2-hydroxy-3-methyl-3-butenyl]-5,6,7-trimethoxycoumarin (Fig. 1B).

As shown in Fig. 1C, both fraction A (200 \( \mu\text{g/ml} \)) and muralatin L (500 \( \mu\text{M} \)) were found to be able to significantly increase the intracellular calcium level in TRPV1-expressing cells, as compared with the positive control capsaicin (5 \( \mu\text{M} \)) that specifically activates TRPV1. The increased calcium signal can be inhibited by TRPV channel blocker ruthenium red (Fig. 1D). These results indicate that the coumarin muralatin L is a novel TRPV1 channel agonist.

### Dose-dependent and Selective Activation of TRPV1 by Muralatin L in HEK-293T Cells

To confirm the effect of muralatin L on TRPV1, we utilized calcium imaging and detected the intracellular fluorescent calcium level in HEK-293T cells expressing TRPV1 channels in response to either test compound or capsaicin. Non-transfected cells showed no elevation of fluorescent calcium as determined by the ratio of Fura-2 in response to either muralatin L (500 \( \mu\text{M} \)) or capsaicin (5 \( \mu\text{M} \)) (Fig. 2A). In contrast, 500 \( \mu\text{M} \) muralatin L induced a

### TABLE 1

| Position | \( \delta_{c} \) (in ppm) | \( \delta_{h} \) (J in Hz) |
|----------|--------------------------|--------------------------|
| 2        | 160.7                    |                           |
| 3        | 113.3                    | 6.18, d (9.7)             |
| 4        | 138.6                    | 7.88, d (9.7)             |
| 5        | 148.0                    |                           |
| 6        | 141.7                    |                           |
| 7        | 155.6                    |                           |
| 8        | 115.6                    |                           |
| 9        | 148.8                    |                           |
| 10       | 109.4                    |                           |
| 1'       | 29.7                     | 3.02, m; 2.92, m          |
| 2'       | 75.0                     | 4.24, m                   |
| 3'       | 147.1                    | 4.71, d (5.6); 4.83, d (5.6) |
| 4'       | 110.3                    | 1.80, s                   |
| 5'       | 17.8                     | 3.93, s                   |
| 5-\text{OCH}_3 | 61.6                 | 3.80, s                   |
| 6-\text{OCH}_3 | 60.7                 | 3.92, s                   |
| 7-\text{OCH}_3 | 61.1                 |                           |

**FIGURE 2.** Muralatin L induces \( \text{Ca}^{2+} \) influx in TRPV1-expressing HEK-293T cells. A, left panels, live-cell fluorescent imaging of untransfected cells (top panels). Right panel, average effects of Fura-2 ratios induced by muralatin L (blue arrow) and capsaicin (red arrow) in untransfected cells (top panel, \( n = 50 \)). B, left image panels, cells expressing hTRPV1 (bottom panels) in response to application of 500 \( \mu\text{M} \) muralatin L or 5 \( \mu\text{M} \) capsaicin. Right panel, average effects of Fura-2 ratios induced by muralatin L (blue arrow) and capsaicin (red arrow) in cells expressing hTRPV1 (bottom panel, \( n = 54 \)). The experiment was repeated three times.
sharp increase of the intracellular calcium fluorescence and a further small increase of calcium fluorescence signal upon addition of 5 μM capsaicin (Fig. 2B), confirming that the elevated calcium resulted from activating the TRPV1 channels. To further confirm the muralatin L effect, we performed whole-cell patch clamp recordings of HEK-293T cells expressing TRPV1 channels. Bath application of muralatin L (300 μM) elicited the activation of TRPV1 current, and the effect could be washed out before further activation of TRPV1 by capsaicin (1.0 μM) (Fig. 3A, left panel). The effect of muralatin L on activating TRPV1 was further confirmed by ramp recordings (Fig. 3A, right panel). Application of different concentrations of muralatin L from 0.3 μM to 3.0 mM resulted in a dose-dependent activation of the TRPV1 current (Fig. 3B). As compared with the potency and maximum effect of capsaicin, fitting the data from dose-dependent activation of TRPV1 by muralatin L with the Hill equation yielded an EC50 value of 205.6 ± 27.4 μM (n = 7) and a Hill coefficient of 1.14, confirming that muralatin L is an activator of TRPV1 with an equal maximum efficacy like capsaicin (Fig. 3C). In addition, we observed no difference between saturated 3 mM muralatin L-induced current and the current from a mixture of 10 μM capsaicin with 3 mM muralatin L. TRPV1 current evoked by 500 μM muralatin L was inhibited by each application of 0.01, 0.1, and 1 μM TRPV1 potent antagonist JNJ-17203212 (Fig. 3E). All these results suggest that muralatin L is a full agonist of TRPV1.
Administration Bureau of Traditional Chinese Medicine, 1999). To test whether muralatin L could activate native TRPV1 currents expressed in DRG sensory neurons where TRPV1 plays a critical role in nociception, we acutely dissociated mouse DRG neurons and performed outside-out patch clamp recordings. As shown in Fig. 5A, a robust TRPV1-like current was elicited upon application of 1 μm capsaicin from DRG neurons (4 of 17), confirming the current was mediated by TRPV1 activation. Membrane patches from DRG neurons were first exposed to 1 μm capsaicin to identify TRPV1-expressing cells before application of muralatin L. Similar to the capsaicin effect, further application of 1 μm muralatin L also resulted in a large TRPV1 current. Repeated applications of muralatin L also resulted in current desensitization (Fig. 6A). These results indicate that muralatin L can activate endogenous TRPV1 current in DRG neurons, likely exerting antinociceptive action by current desensitization.

Reversal of Inflammatory Pain by Muralatin L and Lack of Its Effect in Trpv1 Knock-out Mice—To test the effect of muralatin L on pain, we utilized several rodent models of pain induced by noxious chemicals, acid, or heat. Intraperplantar injection of 1% formalin into either WT or Trpv1 KO mice elicited pain response of mice in two phases as follows: phase I, an early nociceptive response (0–5 min) caused by direct stimulation of C-fiber nociceptors; and phase II, a later second phase of nociceptive behavior (15–30 min) (26), which was observed by the time licking paws (Fig. 7). As shown in Fig. 7A, there was no significant difference between the WT and Trpv1 KO groups in the either phase. In addition, intraplantar injections of either muralatin L (10 mg/kg) or capsaicin (1 μm) into Trpv1 KO mice had no effect on paw licking (Fig. 7A), further confirming that muralatin L, like capsaicin, could not induce pain response in Trpv1-deficient mice. In contrast, inflammatory paw of pain induced by formalin in WT mice, intraperitoneal injection of muralatin L at 10 and 40 mg/kg, caused a dose-dependent decrease of phase II response of inflammatory pain induced by paw injection of formalin about 56% and 95%, respectively, as compared with the group of vehicle control (Fig. 7B).

We also tested the effect of muralatin L on inflammatory pain induced by intraperitoneal injection of acetic acid (1.6%) in mice. As a positive control, injection (intraperitoneal) of morphine (0.2%) caused an analgesic effect by reducing abdominal writhing (Fig. 7C). Similarly, muralatin L resulted in a dose-dependent decrease of writhing and the writhing numbers were decreased about 53% and 78% at the dose of 10 and 40 mg/kg (intraperitoneal), respectively, as compared with the vehicle control (Fig. 7C). To further confirm the effect of muralatin L on writhing induced by intraperitoneal injection of acetic acid, we also evaluated the effect of muralatin L on abdominal writhing in Trpv1 KO mice. The results showed that muralatin L had no further effect on reducing abdominal writhing as compared with the vehicle of Trpv1 KO mice, whereas morphine still caused an analgesic effect (Fig. 7D). These results indicate that muralatin L inhibits the inflammatory pain by directly acting on and desensitizing TRPV1 channel.

In addition, we tested the effect of muralatin L on thermal pain induced by a light beam focused on the middle portion of...
In the thermo-nociceptive test, muralatin L had no effect on reducing thermal pain, as compared with morphine that resulted in reduction of thermo-nociception by increasing latency time (Fig. 7E).

Prediction of TRPV1-binding Sites for Muralatin L by Modeling—The recent cryo-EM structure of rat TRPV1 reveals that there are two constrictions that gate the ion conductance pathway as follows: a funnel-like extracellular pore forming the selectivity filter that functions as an upper gate, and the middle of the S6 helix that constitutes the lower gate where capsaicin binds (27). To explore the binding sites of muralatin L for TRPV1, we docked the muralatin L molecule into the TRPV1 structure using Maestro Suite software (23). The docking results reveal that muralatin L is sequestered in a pocket that is formed by the residues Tyr-511 from S3, Met-547 and Thr-550 from S4, and Glu-570 in the S4-S5 linker of TRPV1. Muralatin L binds to TRPV1 through the C-2\text/H11032-hydroxy group that is critical for a hydrogen bonding interaction with Tyr-511, and the aromatic area and C-3\text/H11032-methyl group interact with the hydrophobic regions being composed of Leu-515, Met-547, Thr-550, Glu-570, and Leu-577 (Fig. 8, B and C), respectively. We also performed a docking analysis of capsaicin with its binding pocket in TRPV1, displaying a similar binding pattern to muralatin L (Fig. 8D). Muralatin L and capsaicin were found to contain two similar pharmacophoric regions by comparing the sequence alignment of the low-gate domains in TRPV1–4 (Fig. 8, E and F). To test the importance of the critical residues identified from the docking, we constructed two mutants of human...
TRPV1 in which Tyr-511 or Thr-550 was substituted by alanine (hTRPV1_{Y511A} and hTRPV1_{T550A}), respectively. The Y511A mutant was functional as determined by activation of 3 mM 2-APB, but it was insensitive to either capsaicin or muralatin L (Fig. 8G), indicating that the residue Tyr-511 that forms the hydrogen bond with muralatin L is critical for muralatin L binding. In contrast, the T550A mutant retained the channel sensitivity to both capsaicin or muralatin L (Fig. 8H). We also tested the effect of muralatin L on other residues such as Leu-515, Leu-547, Glu-570, and Leu-577, that are in close proximity to muralatin L (Fig. 8C). Mutating the individual four residues to alanine retained the robust sensitivity to either capsaicin or 2-APB, and all four mutants, L515A, L547A, E570A, and L577A were also robustly activated by 100 μM muralatin L, suggesting that these residues play a minor role in interacting with muralatin L (data not shown).

**Discussion**

The goal of this study was to identify active components from the genus *Murraya* that are widely used for pain relief as in traditional Chinese medicines and to investigate their molecular mechanisms of action. We isolated muralatin L, a structurally novel coumarin derivative from *M. alata*, through the bioactivity-guided fractionation, and we confirmed that muralatin L specifically activates TRPV1 and reverses inflammatory pain in mice. Our findings not only demonstrate the mechanism of action for muralatin L but also point to the possibility of identifying more structurally diversified natural compounds targeting TRP channels from the flowering plants such as *Murraya* genus.

*Murraya* is a popular genus of the plant family Rutaceae that occurs in tropical and sub-tropical regions of Southeast Asia, China, and northeast Australia. There are nine species and one
FIGURE 8. Putative binding sites for muralatin L in rat TRPV1 channel. A, location of four key residues mapped to the cryo-EM structure of rat TRPV1 (Protein Data Bank code 3J5R). The molecular surface of muralatin L is shown in blue, and key residues are in purple. B, side view of molecular docking of muralatin L into rat TRPV1 using Maestro Suite software. Amino acid side chains of residues Tyr-511, Met-547, Thr-550, and Glu-570 in TRPV1 are shown in pink. The helices are in gray, and the helices of the neighboring monomer are displayed as line ribbons. The ligand is depicted as a blue tube. Hydrogen bonds are black dashed lines. C, representative ichnography of muralatin L interacting with related amino acids of TRPV1. D, docked view of interactions between muralatin L and capsaicin with rat TRPV1 subunits. The binding pocket is composed of residues located in S3, S4, and S5. The key interacting residues are marked and displayed as a thin tube presenting in purple. The chemical structures of muralatin L and capsaicin are shown in blue and red, respectively. E, two compounds are both composed of two pharmacophoric regions as follows: the aromatic A-region and the double bond-participated junction B-region; in addition, capsaicin also possesses a hydrophobic side chain (C-region). F, sequence alignment and comparison of low-gate domains in TRPV1–4. Secondary structure elements are shown above the sequence alignment. Triangles (blue) are indicated as key residues. Current traces of TRPV1 Y511A mutant (G) and TRPV1 T550A mutant (H) expressed in HEK-293T cells in responses to muralatin L or capsaicin or 2-APB.
variety distributed in China, most of which are used as traditional Chinese medicines for treatment of psychogenic pain or somatoform pain disorders (1). For example, the extract of *Murraya paniculata* is used to treat headache, dentalgia, gastalgia, and rheumatalgia, and the 50% aqueous ethanol extract of its root is also clinically used as a topical anesthetic. The extract of *Murraya tetramer*a is regarded as a specific medicine for the management of rheumatic pains at Longzhou District in Guangxi Province in China. Moreover, some of *Murraya* plants have also been developed into drugs such as the prestigious Sanjiuweitai Granula, which is composed of *Murraya exotica*, *Evdia lepta*, *Zanthoxylum nitidum*, etc. for treatment of gastalgia and different kinds of gastritis, including superficial gastritis, erosive gastritis, and atrophic gastritis (28). Because of the widespread use of *Murraya* extracts in traditional medicines, there have been a number of studies investigating the active compounds as well as their mechanisms of action. Zou and co-workers (29) reported the extracts of all *Murraya* species distributed in China for their anti-nociceptive effects on abdominal writhing induced by acetic acid in mice. Among the tested *Murraya* plants, *M. alata* shows the highest inhibitory activity on pain (29). These findings have promoted the further study for identification of their active ingredients. Wu et al. (30) identified six coumarins from the 70% aqueous ethanol extract of *M. exotica*, and one of the isolates, murrarcarpin, exhibits substantial potent activities in anti-nociception and anti-inflammation (31).

Many studies on *Murraya* have been focused on the analgesic effects of the plant extracts as well as the compounds purified from the extracts in animal model tests; however, little is known about the mechanisms underlying the antinociceptive action of *Murraya* plants. Recently, Chen et al. (16) reported a known furanocoumarin, imperatorin, from the *Angelica dahurica* root extract as a new class of partial agonist of TRPV1, exhibiting an EC$_{50}$ value of 12.6 ± 3.2 µM. Imperatorin delays TRPV1 recovery from desensitization and most likely acts via a site adjacent to or overlapping the TRPV1 capsaicin-binding site.

Our primary screening identified the extract from the plant *M. alata* to be the most potent in activating TRPV1 among several *Murraya* extracts, which is consistent with the report by Zou et al. (29). The use of TRPV1 agonists is aimed at achieving desensitization of sensory neurons (8). Topical capsaicin has been used as folk medicine to relieve pain for centuries. Capsaicin is not the only plant-derived agent as the TRPV1 agonist (3) and some other natural products such as eugenol, camphor, evediamine, thymol, and carvacrol can also activate TRPV1 (4, 5, 32, 33). The drawback of using capsaicin, however, is that capsaicin products are associated with an intolerable burning sensation and the need for multiple applications for weeks to mediate their analgesic effects (12). Genetic and pharmacological studies have shown that TRPV1 is an essential component of the cellular signaling mechanisms through which injury produces thermal hyperalgesia and pain hypersensitivity (34–36). The mechanism of pain relief has been proposed to be due to desensitization of the receptor or degeneration of the nerve terminals (37, 38). This phenomenon is a Ca$^{2+}$-mediated process in which Ca$^{2+}$ entry results in a sufficiently high Ca$^{2+}$ concentration to trigger desensitization. Desensitization is now mainly considered to be a dephosphorylation event (39). Through the process of desensitization, a neuron can diminish its overall response to a particular chemical, physical, or electrical signal (38). According to the result from our Fig. 4A, muralatin L induces the desensitization of TRPV1, thus leading to pain relief. There are two likely explanations for the antinociceptive effect of muralatin L. One is that the muralatin L-induced current is reduced in part due to the acute desensitization phase, similar to the effect of capsaicin (Fig. 4C). Imperatorin is an agonist for TRPV1 channel and delays the recovery of TRPV1 from the desensitization (16). Compared with imperatorin, muralatin L as a coumarin derivative shares some structural similarity with imperatorin. Muralatin L also has a similar property with piperine and produces a greater degree of macroscopic desensitization compared with capsaicin (40). The desensitization of the channel prevents continued perception of the stimulus, which makes the investigation of TRPV1 agonists as analgesics for the treatment of chronic pain (41).

Muralatin L is potent in reducing abdominal writhing induced by acetic acid and formalin-induced phase II pain in mice. Formalin is known to evoke nocifensive behaviors in two phases, the first of which is thought to be due to a direct chemoinnocicceptive effect, and the second phase is mainly mediated by inflammatory reactions (26). Our results prove that muralatin L mainly relieves the inflammatory pain. Interestingly, muralatin L has no effect on reducing thermal pain in WT mice. Hot temperature (more than 43 °C) activates TRPV1 channels that cause excruciating pain (3), and Trpv1 knockout mice show dramatic reduction of hypersensitivity to heat during inflammation (35, 36). We observed a similar phenomenon in our tests in which Trpv1 KO mice exhibit a significant reduction of acetic acid-induced pain, and TRPV1-specific muralatin L is ineffective in reducing pain in Trpv1 KO mice, suggesting that TRPV1 is directly involved in this model. However, in a formalin-induced pain model, Trpv1 KO mice show a similar response as WT mice. We speculate that there are two possible explanations. First, mice lacking *trpv1* gene can often exhibit some undefined behaviors. It has been shown that formalin-induced acute chemonociception (or carrageenan-evoked subacute inflammatory mechanical hyperalgesia) remains the same between WT and Trpv1 KO mice, in which the participation of TRPV1 in formalin-induced inflammatory pain can be negligible (42). Moreover, mice lacking thermo-TRPV1 are still able to respond tonoxious heat and show no significant difference in hot plate responses between WT and Trpv1 KO mice (36). The heat threshold of Trpv1 KO mice is not different from that of WT animals, although intraplantar injection of TRPV1 agonist resiniferatoxin induces a profound drop of heat threshold (43). Second, inflammatory pain induced by formalin can be mediated by many factors such as cytokines and cyclooxygenase products besides ion channels (44). Although muralatin L shows selectivity on TRPV1 among the TRP channels tested, we cannot rule out any effect of muralatin L on non-channel targets that are involved in inflammatory pain.
Coumarin Derivative Muralatin L Activates TRPV1

Molecular docking results indicate that muralain L interacts with TRPV1 by a similar fashion as capsaicin, suggesting the resembled action mode for muralatin L and capsaicin in activating TRPV1. Interestingly, muralatin L and capsaicin show some structural similarities. They both possess the aromatic A-region and the double bond-participating junction B-region. In a different way, muralatin L lacks the hydrophobic side chain (C-region) compared with capsaicin, which is a possible reason for its lower activity (Fig. 8E). In future structural modification, lengthening the C-8 substituent group of muralatin L or introducing an appropriate hydrophobic chain at the position of C-2’-OH might result in promoting TRPV1 activation. Taking imperatorin, the previously reported TRPV1 agonist, into consideration, we find that imperatorin is a structurally simple furanocoumarin that possesses an additional furan ring fused at the C-5 and C-6 positions of the coumarin nucleus compared with muralatin L (Fig. 1B), suggesting a feasible entry for the modification of the “A-region” of muralatin L to improve its activity. The docking also provides some hits for the selectivity of muralatin L on TRPV1. By aligning the sequence of low-gate domains of TRPV1 with other TRPV channels, it is noticeable that this part of homology between TRPV members is not well conserved, particularly in the region between S3 and S5. The residues Tyr-511, Met-547, Thr-550, and Glu-570 are clustered at the lower gate. However, TRPV2, TRPV3, and TRPV4 contain distinct residues such as Glu-570 in TRPV2, TRPV3, and TRPV4, as compared with Glu-570 in TRPV1 (Fig. 8F). The Tyr-511 in TRPV1 has been proven to be a critical residue for the action of muralatin L.

Although a weaker agonist compared with capsaicin, muralatin L presents a stronger desensitization than capsaicin upon its repetitive applications and also exhibits considerable efficacy for pain relief in vivo. Thus, muralatin L renders less dosing frequency and likely possesses fewer side effects than the strong TRPV1 activator such as capsaicin. Considering the non-addictive and non-drug-resistant properties of muralatin L as complementary and alternative medicine, we speculate that muralatin L may have a wide potential in pain-related therapy.

In summary, we have demonstrated that the structurally novel coumarin derivative muralatin L, isolated from the M. alata, can selectively activate TRPV1, providing a basis for Murraya extracts as traditional use for pain relief. Our findings also highlight the potential of coumarin analogues for broad pharmaceutical applications, and muralatin L as a second coumarin-based TRPV1 activator possesses the developmental potential for pain therapy.

Author Contributions—N. W. and H. L. conducted the majority of experiments, including isolation of the natural products, FlexStation3 assay, patch clamp recording, calcium imaging, molecular docking, animal behavior tests, and data analysis; Y. W. assisted with fluorescent calcium assay development and rat DRG neuron dissociation; R. L. and S. Y. provided Trpv1 knockout mice for us and supervised animal assays. X. S. conducted FlexStation3 assay. Y. J. supervised molecular docking; N. W., H. L., and Y. W. prepared the draft; K. W. W. and Y. J. conceived and supervised the project, participated in data analysis, and finalized the manuscript writing.

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