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Plumbagin, Juglone, and Boropinal as Novel TRPA1 Agonists

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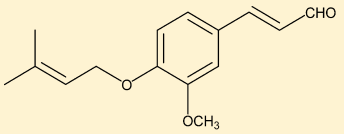
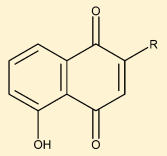
ABSTRACT: A series of seven oxyprenylated phenylpropanoids and naphthoquinones were tested regarding their ability to activate transient receptor potential ankyrin subtype 1 channel (TRPA1). Three of the assayed compounds, namely, boropinal (**3**), juglone (**5**), and plumbagin (**7**), acted as strong modulators of TRPA1 channels with EC_{50} values of 9.8, 1.7, and 0.5 μM, respectively, as assessed by Ca^{2+} assays. Moreover, the compounds elicited TRPA1 currents in electrophysiological whole cell recordings. We additionally provide evidence that plumbagin activated TRPA1-positive neurons isolated from mouse dorsal root ganglion neurons but did not affect sensory neurons from TRPA1-deficient mice. The high potencies of plumbagin and juglone to activate TRPA1 channels may explain the molecular basis of the mucosal irritant properties of these compounds as well as of related naphthoquinones and phytopreparations, as widely reported in the literature.



Boropinal (**3**) EC_{50} = 9.8 μM



R = H Juglone (**5**) EC_{50} = 1.7 μM

R = CH_3 Plumbagin (**7**) EC_{50} = 0.4 μM

The transient receptor potential ankyrin subtype 1 protein (TRPA1) forms a nonselective cation-conducting channel, which is permeable for mono- and divalent cations, including Ca^{2+} and Na^{+}. TRPA1 acts as an irritant sensor, playing a key role in several physiological and pathophysiological functions such as nociception, irritant sensing, and possibly mechanical sensation. TRPA1 is mainly located in nociceptive sensory neurons and in other sensory cells including epithelial cells. The activation of TRPA1 in primary sensory neurons leads to cell membrane depolarization, action potential discharge, and neurotransmitter release at both peripheral and central neural projections.[1] Many potent TRPA1 ligands are cysteine reactive electrophiles. Examples of plant-derived compounds include cinnamaldehyde, curcumin, allyl isothiocyanate (AITC), and oleocanthal. Synthetic substances that activate TRPA1 include farnesyl thiosalicylic acid, formaldehyde, and acrolein. Also nonelectrophilic agents such as menthol, thymol, nicotine, cannabinoids, and PF-4840154 (4-isobutylamino-2-[4-(tetrahydropyran-3-ylmethyl)-piperazin-1-yl]pyrimidine-5-carboxylic acid benzylamide) have been reported to be potent activators of TRPA1. Recent findings indicate that TRPA1 is a promising and valuable target for the treatment of acute and chronic pain, itch, and sensory hyperreactivity in visceral organs such as airways, bladder, and the gastrointestinal tract. The role of TRPA1 in physiology, pathology, and pharmacology has been extensively reviewed by Zygmunt and Högestätt, recently.[2] The listed compounds exhibit a wide range of activities, and some of them display a relatively low potency and poor selectivity, additionally acting with other members of the TRP family.[3] Thus, the search for novel modulators of TRPA1 is a field of current and growing interest.

As a continuation of our ongoing studies, which aim at getting further insights into the pharmacological profiles of naturally occurring oxyprenylated and naphthoquinone secondary metabolites, we decided to investigate whether such compounds affect TRPA1 channels. The chemical structures of the natural compounds we studied are illustrated below. All of them contain an α,β-unsaturated carbon−carbon double bond conjugated to a carboxylic acid or ester and to an aldehyde.

RESULTS AND DISCUSSION

The main natural sources of auraptene (**1**), 4′-geranyloxyferulic acid (**2**), juglone (**5**), lawsone (**6**), and plumbagin (**7**) have been described previously.[4−8] Boropinal (**3**) has been isolated from the aerial parts of the Australian shrub *Boronia pinnata* Sm. (Rutaceae), and nelumal A (**4**) has been extracted from the Chinese medicinal plant *Ligularia nelumbifolia* Hand.-Mazz. (Asteraceae).[9,10]

To investigate the TRPA1-modulating properties of the compounds, we utilized human embryonic kidney HEK 293 cells that were stably transfected with human TRPA1 ($HEK293_{TRPA1}$ cells), which represents a well-validated *in vitro* model to investigate the interaction between chemicals and TRPA1.[11] The compounds were first tested in single calcium imaging using fura-2 as the calcium indicator. All compounds were added at a final concentration of 20 μM followed by the application of the specific TRPA1 channel blocker A-967079 (1 μM).[12] The results are illustrated in Figure 1.

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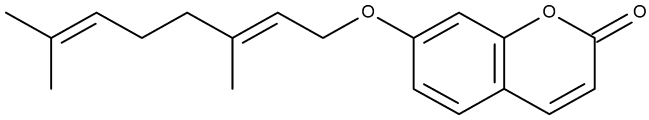

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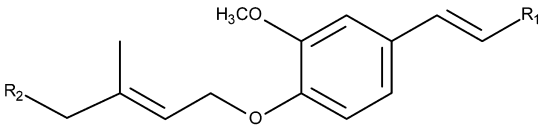
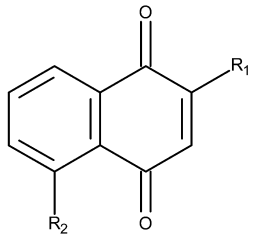


2 R_1 = COOH, R_2 = isopentenyl
3 R_1 = CHO, R_2 = H
4 R_1 = CHO, R_2 = isopentenyl



5 R_1 = H, R_2 = OH
6 R_1 = OH, R_2 = H
7 R_1 = CH_3, R_2 = OH

Boropinal A (**3**), nelumal A (**4**), juglone (**5**), and plumbagin (**7**) elicited increases in $[Ca^{2+}]_i$ in HEK293$_{TRPA1}$ cells but not in control HEK293 cells (Figure 1B, inset). The magnitudes of the calcium responses were similar to the response elicited by the application of the potent TRPA1 channel activator AITC (Figure 1B) and could be largely inhibited by the TRPA1 blocker A-967079. Auraptene (**1**), geranyloxyferulic acid (**2**), and lawsone (**6**) caused only minor increases in $[Ca^{2+}]_i$ when added to the cells. The results concerning plumbagin are in line with a previous report by Muraki and co-workers, which has already provided preliminary data about the interaction of plumbagin (**7**) with TRPA1.[13] We next performed concentration response measurements for the four best activators of the TRPA1 channel using Ca^{2+} assays in fluo-4-loaded HEK293$_{TRPA1}$ cells (Figure 2).

As nelumal A (**4**) exhibited strong inherent fluorescence when excited with light at the wavelength used for fluo-4-assisted Ca^{2+} assays, which interfered with the Ca^{2+} assay, we could not obtain concentration response curves for this compound. Plumbagin was the most effective phytochemical in activating TRPA1, with an approximate EC_{50} of 0.5 μM. However, it was not possible to calculate an accurate EC_{50} due to interfering inherent fluorescence of the compound at concentrations above 3 μM (see rise of the fluorescence signal in HEK293 cells, open circles). Subtracting the nonspecific HEK293 response from the response of the HEK293$_{TRPA1}$ cells yields an EC_{50} of 0.4 μM. Juglone effectively induced a Ca^{2+} signal with an EC_{50} value of 1.7 μM, indicating an even higher potency than AITC (EC_{50} = 3.9 μM). Control HEK293 cells (open circless) did not respond with a Ca^{2+} signal to the addition of juglone. Boropinal was the least potent compound, with an EC_{50} of 9.8 μM.

As TRPA1 channels can also be activated by an elevation in $[Ca^{2+}]_i$,[14] we next tested whether the compounds can elicit TRPA1 currents in whole cell patch clamp recording, in which $[Ca^{2+}]_i$ can be tightly controlled. The measurements revealed that juglone (10 μM) robustly activated inward and outward currents in HEK293$_{TRPA1}$ cells, which could be blocked by A-967079 (Figure 3B). The resulting currents were of the same order of magnitude as AITC-induced TRPA1 currents. In contrast, TRPA1 currents evoked by 1 μM plumbagin were much smaller. Also higher concentrations of plumbagin (10 μM) did not elicit larger currents but led to a minor decrease of the current amplitudes at high voltages (data not shown). Such bimodal action of a TRPA1 agonist with an activation at low and an inhibition at high agonist concentrations has frequently been observed for other TRPA1 agonists, such as nicotine and menthol.[15,16] Boropinal (10 μM) was the least potent compound in activating TRPA1 channels (Figure 3C).

Plumbagin and juglone can both undergo redox cycling and generate hydrogen peroxide, which has been shown to potently activate TRPA1 channels.[17] However, neither the extracellular application of 500−2500 U/mL catalase ($I_{+70\ mV}$ = 65.0 ± 5.2) nor its intracellular application ($I_{+70\ mV}$ = 93.5 ± 17.3) prevented plumbagin-induced TRPA1 currents, indicating that the generation of hydrogen peroxide does not underlie the plumbagin-induced channel activation.

Plumbagin exhibits potent irritant properties,[18,19] which can cause painful irritation when applied externally. We therefore tested whether plumbagin excites sensory neurons isolated from mouse dorsal root ganglia (DRG) via an activation of TRPA1. Approximately 50% of cultured neurons from adult wild-type mice responded to the addition of 10 μM plumbagin with an increase in $[Ca^{2+}]_i$ (Figure 4A and C). Subsequent addition of 10 μM AITC did not activate additional neurons, indicating that plumbagin- and AITC-positive neurons represent the same population of sensory neurons. In contrast, neurons from TRPA1-deficient mice were largely insensitive to the stimulation by plumbagin or AITC (Figure 4B and C), indicating that TRPA1 is the main mediator of immediate Ca^{2+} flux elicited by plumbagin in sensory neurons. Although effective as an TRPA1 agonist, boropinal at a concentration of 10 μM did not induce increases in $[Ca^{2+}]_i$ in TRPA1-positive neurons from wild-type mice (data not shown), possibly reflecting the lower potency of boropinal compared to plumbagin and AITC as a TRPA1 activator.

On the basis of the results obtained with the prenyloxyphenylpropanoids (**1**−**4**) we tested, it can be hypothesized that the presence of an *O*-3,3-dimethylallyl side chain and of an *α*,*β*-unsaturated aldehyde like in boropinal (**3**) and nelumal A (**4**) seem to represent determinant structural characteristics for their TRPA1-activating properties. In fact, auraptene (**1**) and 4′-geranyloxyferulic acid (**2**), which both possess an *O*-geranyl moiety and unsaturated carbon−carbon double bonds conjugated to carboxyl groups, which are notoriously less reactive than an aldehyde toward Michael addition reactions, revealed significantly lower potency. The three naphthoquinones under investigation, juglone (**5**), lawsone (**6**), and plumbagin (**7**), elicited different effects on TRPA1. The lack of a TRPA1-activating effect of lawsone (**6**) under the reported experimental conditions can be explained by the presence of the OH in position 2, which largely decreases the reactivity of the unsaturated double bond as a Michael acceptor by its resonance electron-donating effect, thus preventing the interaction with cysteine residues of TRPA1. Although sharing very close structural similarities, juglone (**5**) and plumbagin (**7**) provided different results regarding their potency, as the presence of the methyl group renders plumbagin a more

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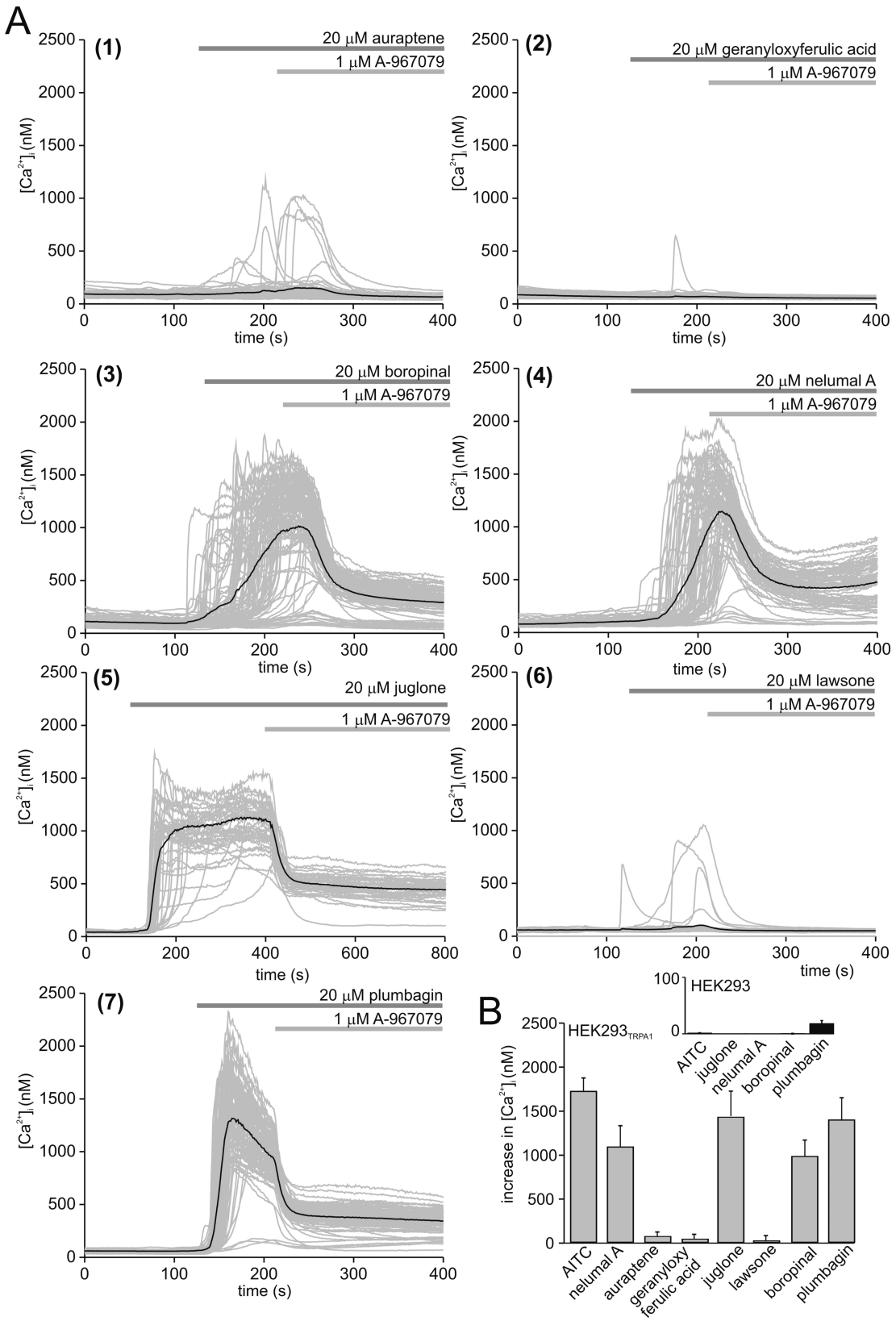

Figure 1. (A) Activation of TRPA1 channels in HEK293$_{TRPA1}$ cells by phytochemicals. Representative single-cell time-lapse analysis of $[Ca^{2+}]_i$ in fura-2-loaded HEK293$_{TRPA1}$ cells, stimulated with test compounds at 20 μM followed by the application of 1 μM A-967079. (B) Statistical analysis of maximal increase in $[Ca^{2+}]_i$ of four independent experiments. Data represent mean ± SEM. Inset depicts increases in $[Ca^{2+}]_i$ in control HEK293 cells without TRPA1 after addition of AITC, juglone, nelumal A, boropinal, and plumbagin.

effective ligand for TRPA1, with an EC_{50} in the nanomolar range and an about 4-fold higher potency compared to juglone. The different logP values reported in the literature, 2.05 for juglone (**5**) and 3.69 for plumbagin (**7**),[18] may partly account

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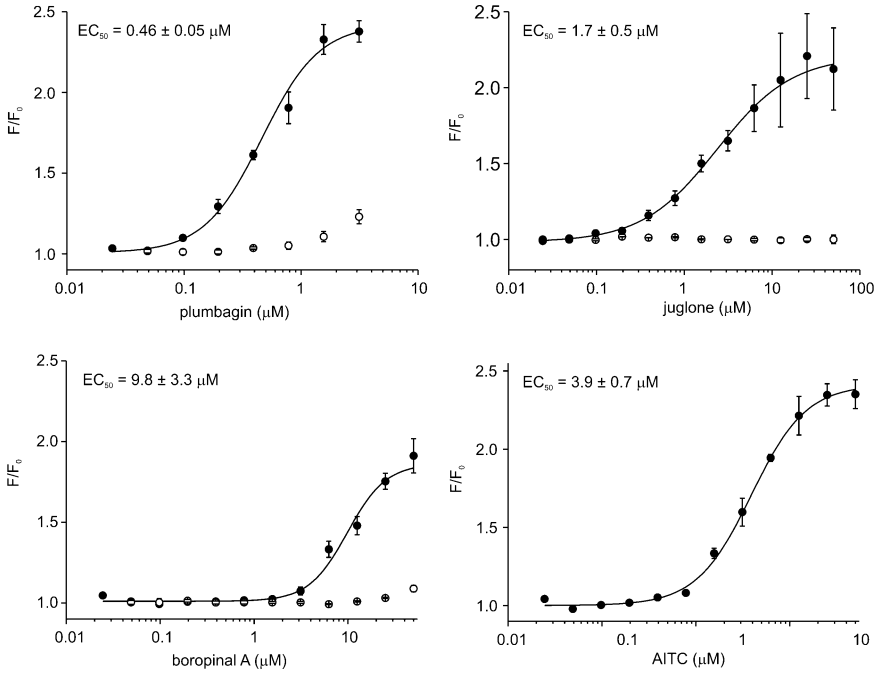

Figure 2. Concentration response curves using fluo-4-loaded HEK293$_{TRPA1}$ cells (solid circles) stimulated with boropinal (**3**), juglone (**5**), plumbagin (**7**), or AITC. Data represent means and SEM of four independent experiments, together with the best fit to a three-parameter Hill equation. Concentration response curves for control HEK293 cells, stimulated with boropinal (**3**), juglone (**5**), or plumbagin (**7**), are shown as open circles.

for these differences. Muraki and co-workers revealed that mutation of the Cys421 in the amino acid sequence of TRPA1 largely decreased the Ca^{2+} response elicited by naphthoquinone diphenyleneiodonium (DPI).[13] Considering the structural similarity between plumbagin and juglone, we assume that a similar mechanism underlies the juglone-induced TRPA1 activation. Alternatively, plumbagin and juglone may undergo redox cycling, thereby generating reactive oxygen species (ROS),[20] which could in turn activate TRPA1. However, we assume that it is unlikely to be the cause of the TRPA1 activation, as neither extra- nor intracellular application of the ROS scavenger catalase did impede plumbagin-induced TRPA1 activation.

Boropinal (**3**) is an *α,β*-unsaturated aldehyde, like many other TRPA1 agonists, and thus its mechanism of action may be explained on the basis of this structural feature. Boropinal has not been thoroughly characterized pharmacologically. In particular, there are no reports on its role in syndromes and pathological conditions in which TRPA1 is believed to be involved. Thus, the data described herein represent a valuable contribution for the further description of pharmacological properties and biomolecular basis of the mechanism of boropinal.

Juglone (**5**), plumbagin (**7**), and phytopreparations containing this 1,4-naphthoquinones are well known for their topical irritant properties and other related adverse effects.[21] Exposure of humans and animals to juglone- and plumbagin-containing plants or their phytopreparations (e.g., dry extracts) may result in painful irritation of the skin, mouth burns, vomiting, diarrhea, hepatic toxicity, increase in white blood cells and neutrophil counts, and a concrete risk of death in the case of ingestion at high doses.

A survey of the literature revealed that the biomolecular basis of juglone- and plumbagin-induced irritation is still not clear. Thus, the action of compounds **5** and **7** on TRPA1 described herein may contribute to our understanding of the mechanism of action of the mucosal irritant properties of juglone, plumbagin, and their plant-derived phytopreparations. In fact, the selective involvement of TRPA1 activation in irritation-based syndromes such as allergic contact dermatitis has been clearly reported in the literature. The pioneering studies, carried out by Jordt and co-workers, largely demonstrated that neuronal TRPA1 channels are necessary to evoke cutaneous inflammation and edema and are tightly linked to both acute and chronic hypersensitivity. The authors also demonstrated that some natural products such as urushiol, a well-known allergen extracted from poison ivy, *Toxicodendron radicans* (L.) Kuntze (Anacardiaceae), oak, *Quercus suber* L. (Fagaceae), and sumac, *Rhus coriaria* L. (Anacardiaceae), are TRPA1 agonists and can elicit a topical inflammatory response.[22]

The search for novel modulators of the TRPA1 receptor is still a challenging and stimulating field of research. We investigated the effects of two selected groups of naturally occurring compounds, oxyprenylated phenylpropanoids and 1,4-naphthoquinones, both having an *α,β*-unsaturated carbon−carbon double bond as a common structural feature. Our results indicate that phenylpropanoids having the aldehyde function and an isopentenyloxy side chain (e.g., boropinal (**3**)) and naphthoquinones (e.g., juglone (**5**) and plumbagin (**7**)) displayed potency equal or superior to the known ligand AITC. Thus, both groups may represent lead compounds for a novel class of TRPA1 agonists.

EXPERIMENTAL SECTION

Chemicals. Auraptene (**1**), 4′-geranyloxyferulic acid (**2**), boropinal (**3**), and nelumal A (**4**) were synthesized as described previously[10,23] (purity ≥97.6% for all compounds assayed by HPLC) and used after NMR characterization. Analytical data for compounds **1**−**4** were in full agreement with those previously reported.[10,23] AITC, juglone (**5**),

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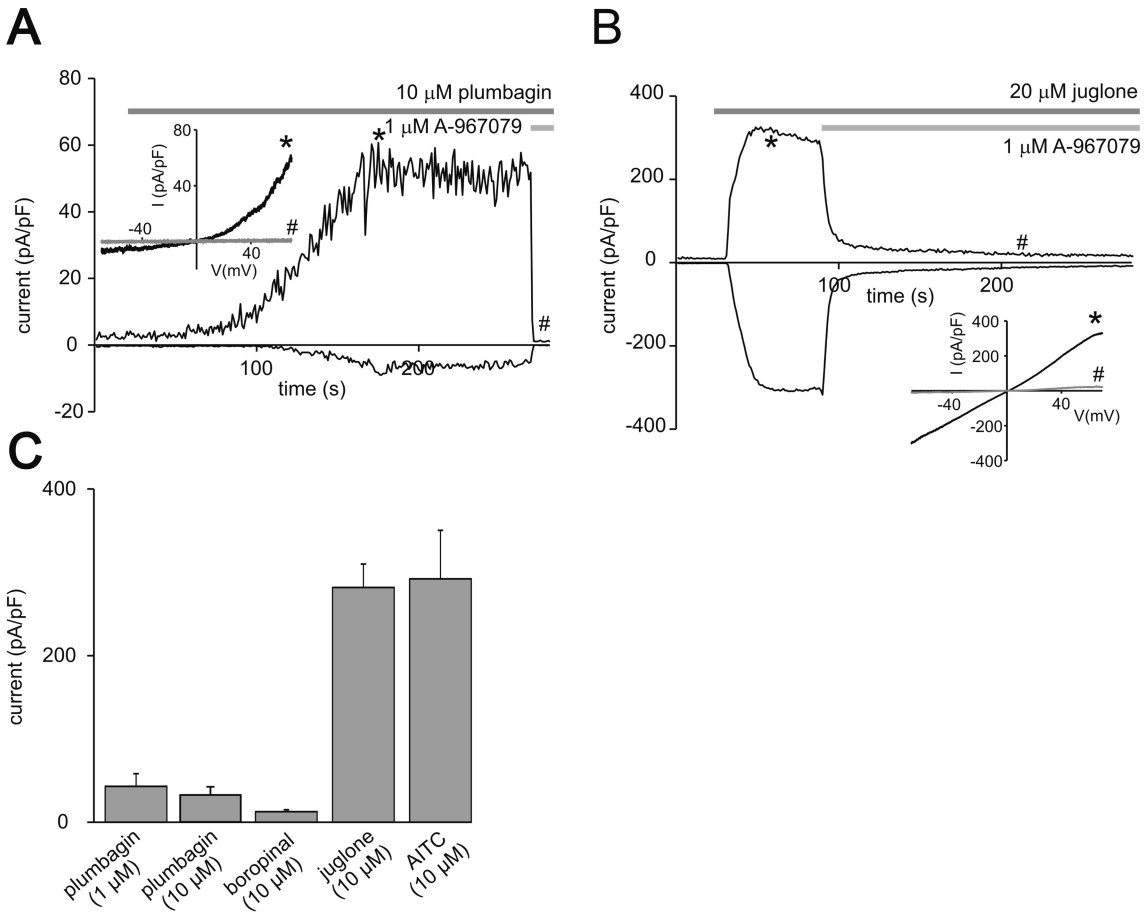

Figure 3. Plumbagin-, boropinal-, and juglone-induced TRPA1 currents as assessed by whole cell patch clamp recordings. (A) Time-lapse analysis of an example recording. A 1 μM plumbagin solution was added to the extracellular solution, followed by the addition of 1 μM A-967079 at the time points indicated by the bars. Data were extracted from voltage ramps (as shown in the inset), ranging from −70 to +70 mV. (B) Similar recording to those in B with 10 μM juglone added to the extracellular solution, followed by addition of 1 μM A-967079. (C) Statistical analysis of several experiments such as in A and B, demonstrating TRPA1 currents in response to plumbagin (1 and 10 μM), boropinal (10 μM), juglone (10 μM), and AITC (10 μM). Currents were normalized to the cell capacity and are depicted as current densities.

lawsone (**6**), and plumbagin (**7**) were purchased from Sigma-Aldrich Chemical Co. and used without further purification.

Cell Culture. HEK293 cells stably transfected with human TRPA1 ($HEK293_{TRPA1}$) were cultured in Eagle's minimum essential medium (Biochrom, Berlin, Germany), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 1 mg/mL geneticin (G418) (Invitrogen, Carlsbad, CA, USA) as described previously.

Preparation of DRG Neurons. $TRPA1^{-/-}$ mice were purchased from Charles River Laboratories. Adult male and female animals were used for this study. Mice were sacrificed by an overdose of CO_2, and DRG neurons were prepared as described.[24] Neurons were maintained in Dulbecco's modified Eagle's medium (Biochrom), supplemented with 30 mM glucose, 2.5 mM L-glutamine, 15 mM HEPES, 0.05 mg/mL gentamicin, 20% FCS, 100 ng/mL nerve growth factor, and 1% ITS liquid media supplement. Cells were cultured for 24−48 h prior to experiments.

Intracellular Ca^{2+} Analysis. To generate concentration response curves, intracellular Ca^{2+} measurements were conducted in 384-well microtiter plates (10 000/well; Corning Inc., Corning, NY, USA) using a custom-made fluorescence imaging plate reader as described previously.[24] In brief, cells were incubated with 5 μM fluo-4/AM (Invitrogen, USA) in HEPES-buffered solution (HBS; 10 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose) for 30 min, rinsed in HBS solution, and seeded into microtiter plates (10 000/well). Cells were stimulated with the compounds, and increases in the intensity of fluo-4 fluorescence were measured. For analysis of single-cell $[Ca^{2+}]_i$, $HEK293_{TRPA1}$ cells were seeded onto glass coverslips coated with poly-L-lysine. Twenty-four to 48 h after plating, cells were loaded with 2 μM fura-2/AM (Molecular Probes, Eugene, USA) for 30 min at 37 °C in HBS buffer and submitted to measurements as described previously.

Electrophysiological Recordings. For electrophysiological recordings, $HEK293_{TRPA1}$ cells were seeded onto poly-L-lysine-coated glass coverslips 24 h prior to experiments. Experiments were performed at room temperature, using a multiclamp 700B amplifier with a Digidata 1440A digitizer under the control of pClamp 10 software. The extracellular recording solution consisted of 140 mM NaCl, 5 mM CsCl, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (adjusted to pH 7.4 with NaOH). Extracellular Ca^{2+} was omitted from the extracellular solution to prevent Ca^{2+}-induced potentiation and run-down of TRPA1 channel activity. Standard intracellular solution contained 140 mM CsCl, 4 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES (adjusted to pH 7.2 with CsOH). Serial resistances were <10 MΩ and were compensated by 80%. Whole cell currents were filtered at 3 kHz (four-pole Bessel filter) and sampled at 10 kHz. Catalase was purchased from Sigma-Aldrich. For removal of thymol, catalase aliquots were centrifuged at 13000*g* for 5 min, washed with 50 mM phosphate buffer, and resuspended in the same volume with phosphate buffer.

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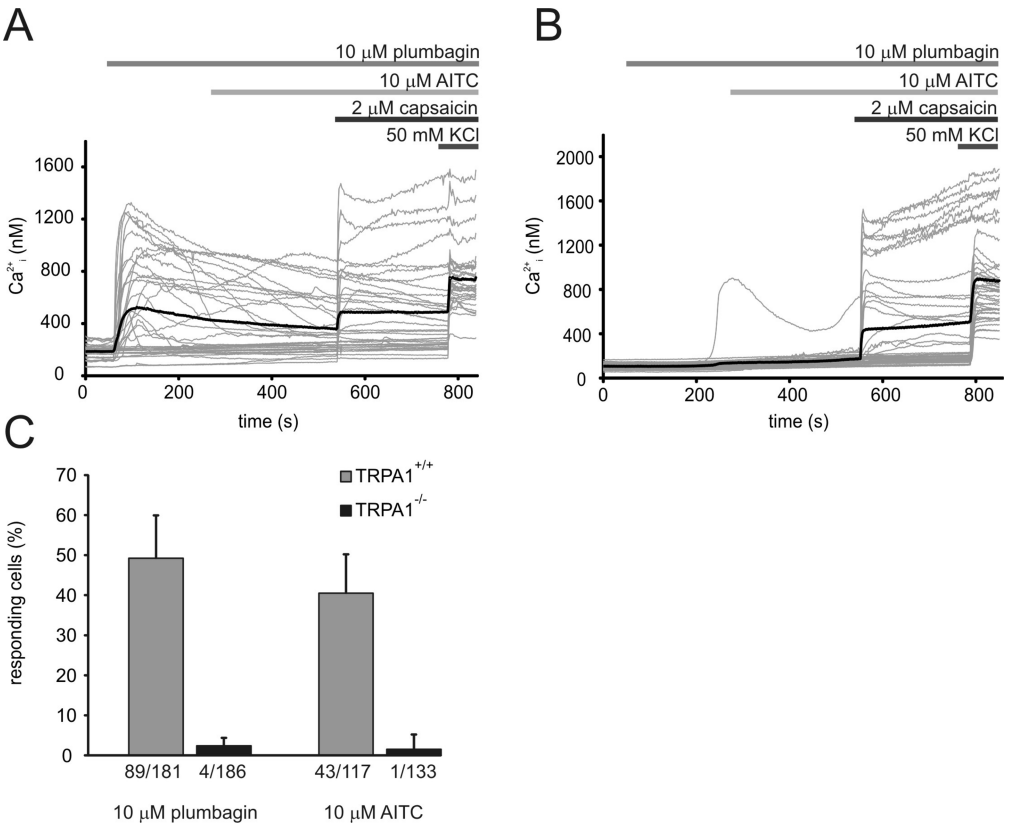

Figure 4. Plumbagin-induced influx in DRG neurons from TRPA1$^{+/+}$ and TRPA1$^{-/-}$ mice. (A, B) Representative time-lapse analyses of fura-2-loaded DRG neurons from TRPA1$^{+/+}$ (A) and TRPA1$^{-/-}$ (B) mice after addition of plumbagin, followed by AITC, capsaicin, and KCl, to select neuronal cells expressing voltage-sensitive Ca^{2+} channels. (C) Statistical analysis of several (n > 5) independent experiments such as in A and B, depicting percentage of plumbagin- and AITC-sensitive cells, isolated from TRPA1$^{+/+}$ (gray) and TRPA1$^{-/-}$ (black) mice.

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Notes

The authors declare no competing financial interest.

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