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Synthesis of resveratrol derivatives as new analgesic drugs through desensitization of the TRPA1 receptor

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ABSTRACT

A series of 31 resveratrol derivatives was designed, synthesized and evaluated for activation and inhibition of the TRPA1 channel. Most acted as activators and desensitizers of TRPA1 channels like resveratrol or allyl isothiocyanate (AITC). Compound **4z** (HUHS029) exhibited higher inhibitory activity than resveratrol with an IC_{50} value of 16.1 μM. The activity of **4z** on TRPA1 was confirmed in TRPA1-expressing HEK293 cells, as well as in rat dorsal root ganglia neurons by a whole cell patch clamp recording. Furthermore, pretreatment with **4z** exhibited an analgesic effect on AITC-evoked TRPA1-related pain behavior in vivo.

Resveratrol (**1**) is a natural phenol and phytoalexin that consists of two aromatic rings (A and B rings) attached by an ethenyl moiety (Fig. 1). Resveratrol was first isolated from the roots of *Polygonum cuspidatum*, a plant used in traditional Chinese medicine,[1] and has been reported to have beneficial effects on human health such as anti-inflammatory, antioxidant, anti-tumor, anti-aging, cardiovascular protective and chemoprotective bioactivity.[2] In addition, analgesic properties of resveratrol against pain triggered by nocifensive stimuli, inflammation, or nerve injury have been demonstrated.[3] The molecular target of resveratrol had been elusive until our group discovered that resveratrol modulates transient receptor potential A1 (TRPA1) channels.[4]

TRPA1 is expressed by a subset of small-sized dorsal root ganglia (DRG) or trigeminal ganglia neurons.[5] In addition to cold, many noxious compounds including allyl isothiocyanate (AITC), cinnamaldehyde, cannabinoids, and allicin activate TRPA1.[6] TRPA1 is also activated by endogenous molecules such as bradykinin, intracellular alkalization, and 4-hydroxynonenal.[6a,7] Pharmacological or genetic block of the channels has demonstrated that TRPA1 is an important component of the transduction machinery that elicits inflammatory and neuropathic pain.[8] This evidence indicates that TRPA1 is a critical molecule for the detection and modulation of pain sensations.

In our previous report, resveratrol concentration-dependently suppressed the AITC-induced currents (I_{AITC}) in HEK293 cells that express TRPA1 with an IC_{50} value of approximately 0.75 μM.[4] Nalli

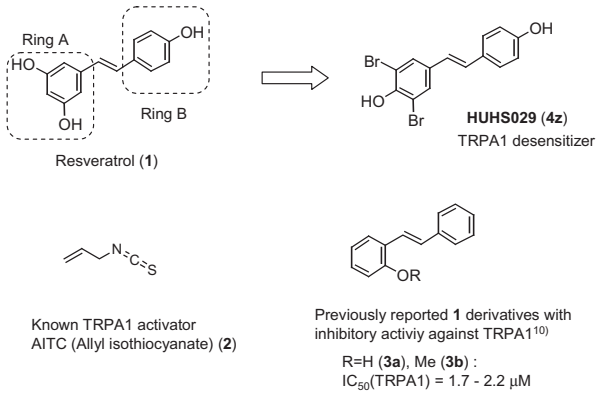

Fig. 1. Structures of resveratrol (**1**), AITC (**2**), and previously reported TRPA1 inhibitors (**3**).

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[e] A.T. and Y. D. contributed equivalently to this work.

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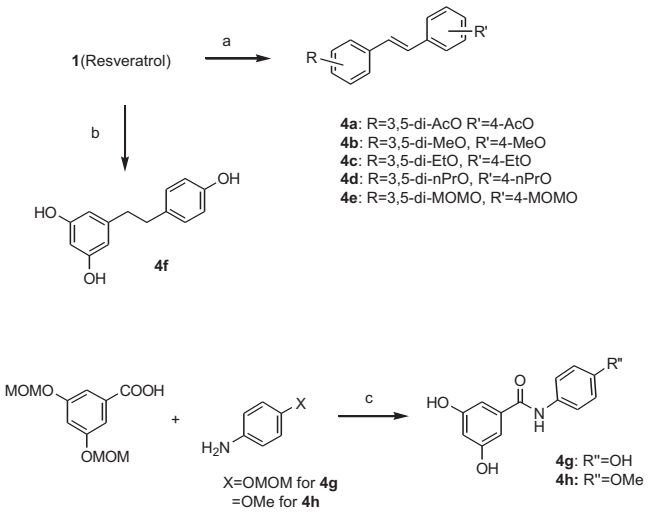
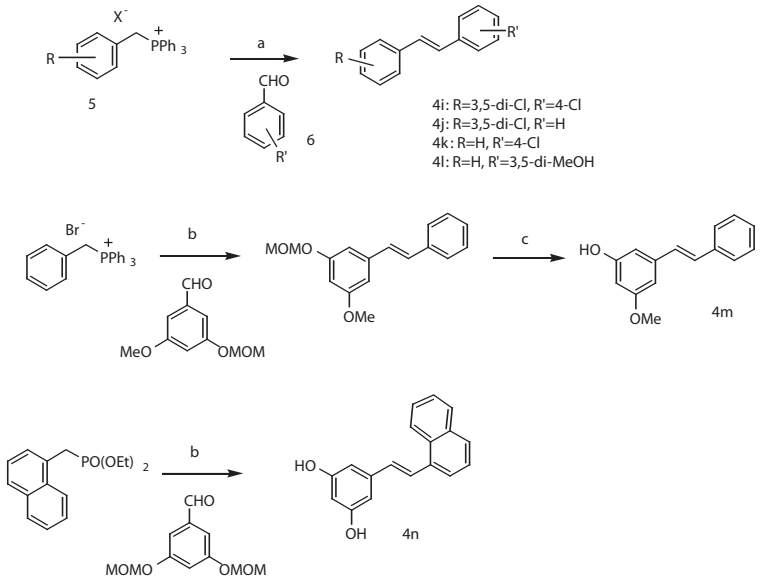


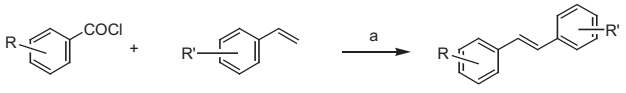
Scheme 1. Synthesis of compounds **4a**–**4h**. Reagents and conditions: (a) Ac_2O, Py for **4a**, MeI, K_2CO_3 for **4b**, EtBr, KI, K_2CO_3 for **4c**, nPrI, K_2CO_3 for **4d**, and $MeOCH_2$-Cl (MOM-Cl), DIPEA for **4e**, (b) H_2/Pd-C, (c) (i) EDC hydrochloride, DMAP, (ii) 4 N HCl/AcOEt.

et al. have reported simple modifications of resveratrol and the activities of the resulting analogues against TRPA1.[9] To further explore resveratrol analogues with better bioactivity against TRPA1, we designed, synthesized, and evaluated in detail 31 resveratrol derivatives for their modulatory effect on the TRPA1 channel.

Resveratrol derivatives (**4a**–**4ee**) were synthesized according to methods described in previous papers[10,11] (Schemes 1–4). Compounds **4a**–**4f** were directly synthesized from resveratrol (**1**), and amide derivatives (**4g**–**4h**) were obtained by amide coupling reactions of the corresponding amines and carboxylic acids (Scheme 1). Compounds **4i**–**4n** were obtained from the corresponding Wittig reagents (**5**) and benzaldehydes (**6**) (Scheme 2). Compound **4n** was similarly prepared by a Wittig-Horner reaction. Synthesis of compounds **3b** and **4o**–**4ee** is shown in Scheme 3. Coupling of acid chlorides (**7**) with substituted styrenes (**8**) was carried out using a palladium-catalyzed decarbonylative Heck reaction with continuous demethylation with BBr_3 or deacetylation with aqueous solution of NaOH to give **4o**–**4ee**. Compounds **4ff**–**4gg** were also prepared by the palladium-catalyzed decarbonylative Heck coupling, and biphenyl derivative **4hh** was obtained by a palladium-catalyzed Suzuki coupling (Scheme 4).



Scheme 2. Synthesis of compounds **4i**–**4n**. Reagents and conditions: (a) *n*-BuLi (X = Br) for **4i**, *n*-BuLi (X = Cl) for **4j**, *n*-BuLi (X = Br) for **4k**, *n*-BuLi (X = Cl) for **4l**, (b) *n*-BuLi, (c) 4 N HCl/AcOEt.

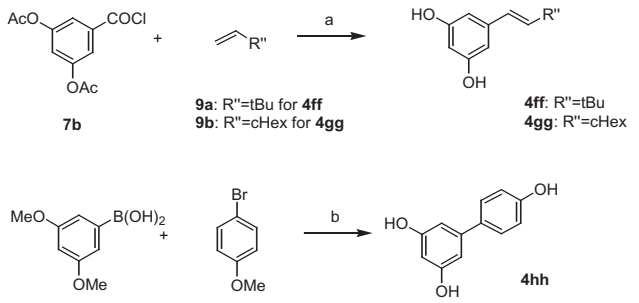


7a: R=H for **3b**
7b: R=3,5-di-AcO for **4o-x**, **4bb-4ee**
7c: R=3,4-di-MeO for **4y**
7d: R'=3,5-di-Br-4-MeO for **4z**, **4aa**

8a: R'=4-MeO for **4o**
8b: R'=H for **4p**
8c: R'=4-Cl for **4q**
8d: R'=4-EtO for **4r**
8e: R'=4-nPrO for **4s**
8f: R'=4-nHexO for **4t**
8g: R'=4-cHexCH_2O for **4u**
8h: R'=4-BzlO for **4v**
8i: R'=2-MeO for **4w**, **3b**
8j: R'=3-MeO for **4x**
8k: R'=4-AcO for **4y**, **4z**
8l: R'=3,5-di-MeO for **4aa**
8m: R'=3,4-OCH_2O- for **4bb**
8n: R'=3,4-di-MeO for **4cc**, **4dd**
8o: R'=3,4,5-tri-MeO for **4ee**

3b: R=H, R'=2-MeO
4o: R=3,5-di-HO, R'=4-MeO
4p: R=3,5-di-HO, R'=H
4q: R=3,5-di-HO, R'=4-Cl
4r: R=3,5-di-HO, R'=4-EtO
4s: R=3,5-di-HO, R'=4-nPrO
4t: R=3,5-di-HO, R'=4-nHexO
4u: R=3,5-di-HO, R'=4-nHexCH_2O
4v: R=3,5-di-HO, R'=4-BzlO
4w: R=3,5-di-HO, R'=2-HO
4x: R=3,5-di-HO, R'=3-HO
4y: R=3,4-di-HO, R'=4-HO
4z: R=3,5-di-Br, 4-HO, R'=4-HO
4aa: R=3,5-di-Br, 4-HO, R'=3,5-di-HO
4bb: R=3,5-di-HO, R'=3,4-OCH_2O-
4cc: R=3,5-di-HO, R'=3,4-di-MeO
4dd: R=3,5-di-HO, R'=3,4-di-HO
4ee: R=3,5-di-HO, R'=3,4,5-tri-HO

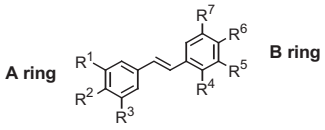
Scheme 3. Synthesis of compounds **4o**–**4ee**. Reagents and conditions: (a) (i) $Pd(OAc)_2$, *N*-ethylmorpholine, (ii) 1 N NaOH for **4o**–**4v** and **4bb**–**4cc**, BBr_3 for **4w**–**4aa**, and **4dd**–**4ee.**



Scheme 4. Synthesis of compounds **4ff**–**4hh**. Reagents and conditions: (a) (i) Pd $(OAc)_2$, *N*-ethylmorpholine, (ii) 1 N NaOH, (b) (i) $Pd(PPh_3)_4$, Na_2CO_3, (ii) BBr_3.

We evaluated compounds for their inhibitory activity of the TRPA1 receptor by the increase in concentration of free Ca^{2+} in HEK293 cells expressing the hTRPA1 receptor after stimulation with AITC using the Calcium Kit II-Fluo4 (Table 1). Desensitization of receptors is a fundamental approach for reducing TRPA1 channel activation. Therefore, the effects of pretreatment with the compounds in TRPA1-expressing HEK293 cells on calcium influx, an agonistic effect, were also examined (Table 1). As shown in Table 1, all compounds almost equally stimulated TRPA1 channel (relative activity: 0.3–0.7) and among of those that had inhibitory activity, they might be desensitizers such as resveratrol against AITC. In this study, all compounds, including resveratrol (**1**), the previously reported resveratrol derivative (**3a**), and **4a**–**4ii**, were assessed first at 30 μM (Tables 1 and 2). Compound **3a**[9] showed 1.1 times more potent inhibitory activity than resveratrol. Inhibitory activities were reduced by acetylation or alkylations of the phenolic hydroxyl groups (**4a**–**4e**). Modification of the hydroxyl group of ring B of **1** (**4p**–**4o**, **4m**–**4q**, **4w**–**4bb**) also decreased the activities. Among them, compound **4x**, bearing a hydroxyl group at the 3 position, showed relatively potent inhibitory activity. Replacement of the hydroxyl group of **1** on ring B with a chlorine atom maintained activity (**4q**), which indicated the hydroxyl group was not vital for the inhibition. Replacement of the hydroxyl groups with chlorine atoms was therefore carried out on ring A as well (**4i**–**4k**). Compounds **4i** and **4j**, bearing 3,5-dichloro atoms on ring A, exhibited relatively potent inhibitory activity as expected. Addition of a hydroxyl group on ring B (**4ee**), methylation of the hydroxyl group of **3a**, and removal of a hydroxyl group of ring A of 1 decreased

Table 1
Structures of resveratrol derivatives with modifications of the hydroxy groups of **1** and TRPA1 effective and inhibitory activities.



	A ring			B ring				Relative inhibitory activity at 30 μM*)	Relative agonisticactivity at 30 μM**)
	R^1	R^2	R^3	R^4	R^5	R^6	R^7		
1 (Resveratorol)	OH	H	OH	H	H	OH	H	1	0.4
3a	H	H	H	OH	H	H	H	1.1	0.6
3b	H	H	H	OMe	H	H	H	0.3	0.4
4a	OAc	H	OAc	H	H	OAc	H	0.5	0.5
4b	OMe	H	OMe	H	H	OMe	H	0.3	0.3
4c	OEt	H	OEt	H	H	OEt	H	0.4	0.4
4d	O-nPr	H	O-nPr	H	H	OnPr	H	-0.1	0.3
4e	OMOM	H	OMOM	H	H	OMOM	H	0.4	0.5
4p	OH	H	OH	H	H	H	H	-0.1	0.4
4x	OH	H	OH	H	OH	H	H	0.8	0.4
4o	OH	H	OH	H	H	OMe	H	0.3	0.3
4l	OMe	H	OMe	H	H	H	H	0.5	0.6
4m	OH	H	OMe	H	H	H	H	0.6	0.7
4r	OH	H	OH	H	H	OEt	H	-0.2	0.3
4s	OH	H	OH	H	H	OnPr	H	0.1	0.3
4t	OH	H	OH	H	H	OnHex	H	0.1	0.5
4u	OH	H	OH	H	H	OCH2cHex	H	-0.1	0.4
4v	OH	H	OH	H	H	OBzl	H	0.7	0.3
4q	OH	H	OH	H	H	Cl	H	0.5	0.3
4i	Cl	H	Cl	H	H	Cl	H	0.8	0.3
4j	Cl	H	Cl	H	H	H	H	0.7	0.3
4k	H	H	H	H	H	Cl	H	-0.2	0.3
4w	OH	H	OH	OH	H	H	H	0.6	0.4
4dd	OH	H	OH	H	OH	OH	H	0	0.3
4cc	OH	H	OH	H	OMe	OMe	H	0.4	0.3
4bb	OH	H	OH	H			H	0.6	0.3
4ee	OH	H	OH	H	OH	OH	OH	0.4	0.3
4ii	H	H	H	H	H	OH	H	0.1	0.4
4y	OH	OH	H	H	H	OH	H	-0.3	0.4
4z (HUHS029)	Br	OH	Br	H	H	OH	H	1.4	0.5
4aa	OH	H	OH	H	Br	OH	Br	0.8	0.3

*) Inhibitory activity of resveratrol = 1.0.
**) Effective activity of AITC = 1.0.

Table 2
Structures of 3,5-dihdroxyphenyl-5-substituted derivatives and TRPA1 inhibitory activities.

		Relative inhibitory activity at 30 μM[*)]	Relative agonistic activity at 30 μM[**)]
4f	-CH_2CH_2-(4-HO)Ph	1.2	0.4
4g	-CONH-(4-HO)Ph	-0.6	0.3
4h	-CONH-(4-MeO)Ph	-0.4	0.3
4n	(E) -CH=CH-α-Naphthyl	1.1	0.3
4gg	(E)-CH=CH-cHex	0.5	0.3
4ff	(E)-CH=CH-*tert* Butyl	0.8	0.3
4hhh	-(4-HO)Ph	0.8	0.5

[*)] [**)] See footnotes of Table 1.

Table 3
Concentration-dependent activities of selected compounds in TRPA1 inhibition.

	Inhibitory value (%)				
	3 μM	10 μM	30 μM	IC_{50} (μM)[*)]	EC_{50} (μM)[**)]
1 (Resveratrol)	−3.8	2.5	37.5	>30	>30
3a	−1.9	−5	−9.5	>30	>10
4x	−19.7	−17.8	1.4	>30	>30
4q	6.1	5.6	18.6	>30	>30
4i	3	7.2	20.6	>30	>30
4j	−16.3	6.2	8.4	>30	>30
4z (HUHS029)	14	37.3	66.7	16.1	>10
4f	−12.7	1.2	−10.2	>30	>30
4h	−5.9	−8.2	11.8	>30	>30

[*)] Inhibitory activity in the TRPA1 assay.
[**)] Agonistic activity in the TRPA1 assay.

inhibitory activities. The compound bearing 3,4-dihydroxy groups on ring A (**4y**) had diminished inhibitory activity. Replacement of the 3,5-dihydroxyphenyl of 1 with 3,5,-dibromo-4-hydroxyphenyl (**4z**) resulted in 1.4 times increased the inhibitory activity, while replacement of the 4-hydroxyphenyl with a 3,5-dibromophenyl moiety (**4aa**) did not affect the activity. Replacement of the 2-hydroxy group of **3a** with a 4-hydroxy group (**4ii**) decreased the relative inhibitory activity while the relative effective activity was almost the same. 5-Substituted resorcinol derivatives were also synthesized (Table 2). Reduction of double bond of **1** increased the inhibitory activity (**4f**). Replacement of the double bond of **1** with an amide bond (**4g**–**4h**) and removal of that group (**4hh**) abolished the inhibitory activity. Replacement of the 4-hydroxyphenyl with a 1-naphthyl moiety (**4n**) gave 1.1 times increased the activity while those with aliphatic moieties (**4gg**–**4ff**) had reduced activities. Among these compounds, compound **4z** (HUHS029) exhibited the more potent inhibitory activity than resveratrol (**1**) and previously reported compounds (**3a**–**3b**).

We next measured concentration-dependent activities of compounds **1**, **3a**, **4x**, **4q**, **4i**, **4j**, **4z**, **4f**, and 4n (Table 3). Among them, compound **4z** exhibited the most potent inhibitory activity with an IC_{50} value of 16.1 μM, while reported TRPA1 inhibitors such as **1** and **3a** had IC_{50} values of more than 30 μM in our assay. Therefore, we selected **4z** (HUHS029) for further study and analyzed its effects on the electrophysiological properties of the TRPA1 channel and AITC-evoked pain behavior (Figs. 3–5).

Using a cell-based calcium assay, we obtained a concentration-response curve for **4z** and AITC-induced calcium influx in TRPA1-transfected-HEK293 cells (Fig. 2). The EC_{50} of **4z** on TRPA1 was 29.9 μM, which was 4-fold higher than that of AITC. The maximum responses of **4z** and AITC were almost equal (Fig. 2), indicating that

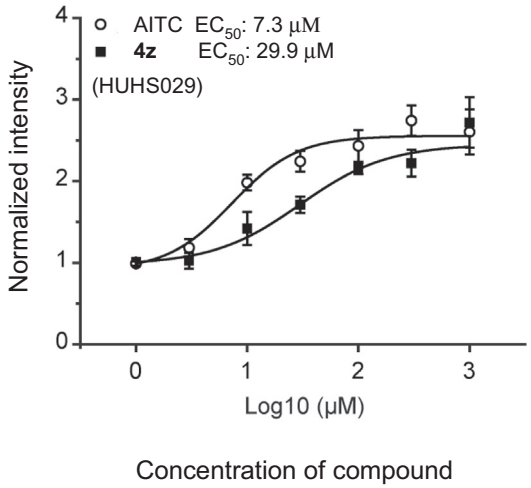


Fig. 2. Concentration–response curve for AITC (open circles) and compound **4z** (filled squares)-induced calcium influx assays in TRPA1-expressing HEK293 cells.

4z was a full agonist for TRPA1. To further determine the agonistic action of **4z** on TRPA1 channels, activated currents in TRPA1-transfected HEK293 cells elicited by this compound were examined (Fig. 3). HUHS029 (**4z**) did not induce any current in untransfected HEK293 cells as measured by voltage clamp recordings (Fig. 3A). In contrast, **4z** induced significant inward currents in TRPA1-transfected HEK293 cells in a concentration-dependent manner (Fig. 3B, C). The activated currents of **4z** were blocked completely and reversibly by HC-030031, a TRPA1-selective antagonist (Fig. 3B).

Pharmacological desensitization of receptors is a fundamental approach for reducing channel activation. The effects of pretreatment of **4z** on the AITC-induced currents in TRPA1-expressing HEK293 cells in a Ca^{2+}-containing extracellular solution were examined. A single application of 300 μM AITC (without **4z** pretreatment) induced robust inward currents with a current density of −121.3 ± 42.2 pA/pF (Fig. 3D, F), which were attenuated significantly by pretreatment of **4z** in a concentration-dependent manner (Fig. 3E, F). These data indicated that **4z** can inhibit AITC-activated TRPA1 channel activation through channel desensitization, which was supported by the above findings in the calcium assay (Table 3). In the series of the patch clamp experiments, we found that **4z** at 5 μM induced smaller inward currents by itself but caused a larger inhibition on AITC-induced current (Fig. 3C and F), suggesting that appropriate dosage of **4z** might alleviate TRPA1-related pain without striking nociceptive effects.

HUHS029 (**4z**) was demonstrated to activate TRPA1-expressing HEK293 cells in the present study. Next, we raised the question as to whether **4z** could activate sensory neurons. Cultured DRG neurons from Sprague-Dawley rats were used to answer the question. TRPA1 is thought to be co-expressed with TRPV1 in DRG neurons.[8] AITC application followed by a capsaicin application, which can activate TRPV1, to the DRG neurons has often been used to detect TRPA1 currents.[4,12] Our data showed that **4z** (30 μM) evoked currents in rat DRG neurons, which were also activated by AITC (300 μM) and capsaicin (1 μM) (Fig. 4).

Activation of TRPA1 by pungent natural products suggests a nociceptive role for TRPA1. Desensitization of TRPA1 channels was expected to be a therapeutic approach in pain relief. Due to its agonism, **4z** activated and then desensitized TRPA1 in the *in vitro* experiments. To further examine if **4z** could also suppress the TRPA1-mediated pain behavior *in vivo*, we administered intraplantar injections of **4z** followed by AITC injections to Sprague-Dawley rats and recorded the AITC-induced nocifensive

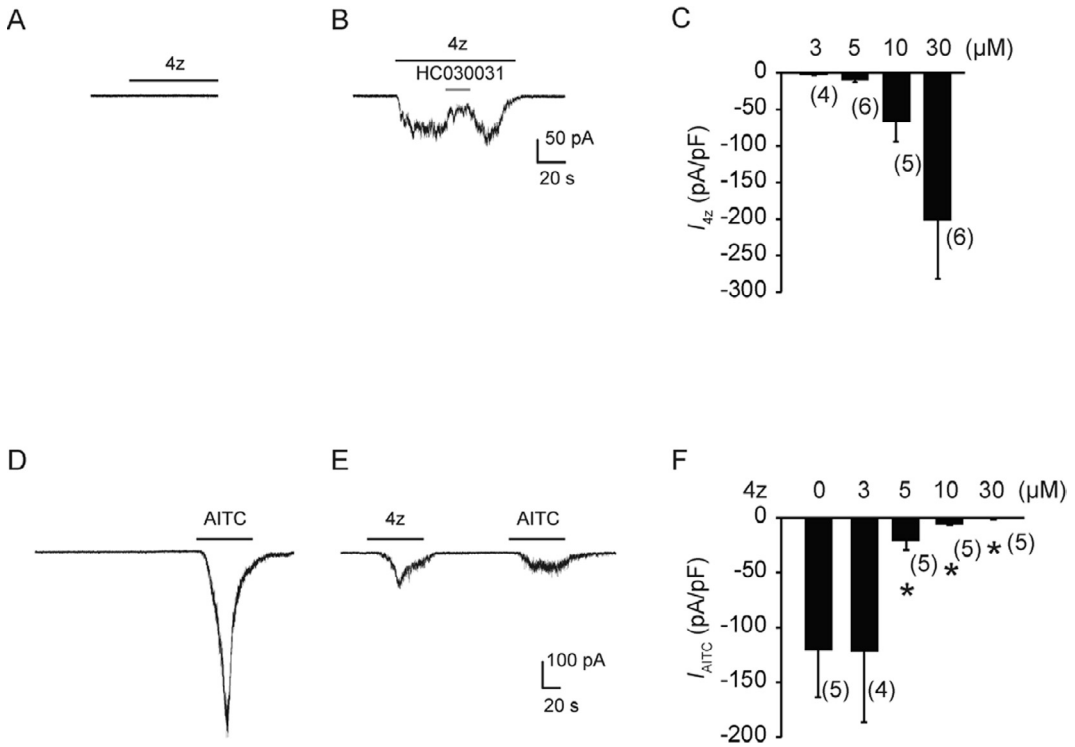


Fig. 3. Compound **4z**-induced activation and desensitization of TRPA1 in heterologous TRPA1-expressing HEK293 cells. (A) Representative traces for **4z** (10 μM)-induced activation in native HEK293 cells. (B) Representative traces for **4z** (10 μM)-induced activation in TRPA1-expressing HEK293 cells. HC-030031 (10 μM), a TRPA1 selective antagonist, inhibited the **4z**-induced TRPA1 current. (C) Concentration -dependency of **4z**-induced TRPA1 activation. (D) Representative traces for the AITC (30 μM, 1 min)-induced TRPA1 currents in the absence of **4z**, (E) in the presence of **4z** (5 μM, 1 min) pre-application. (F) Concentration-dependency of current densities of AITC (30 μM) in the absence or presence of **4z** (3 μM,5 μM, 10 μM or 30 μM) pre-application. $^{*}p < 0.05$, vs. AITC without **4z** pre-application. The results are shown as the means ± SEM, Numbers of the tested cells are shown in parenthesis.

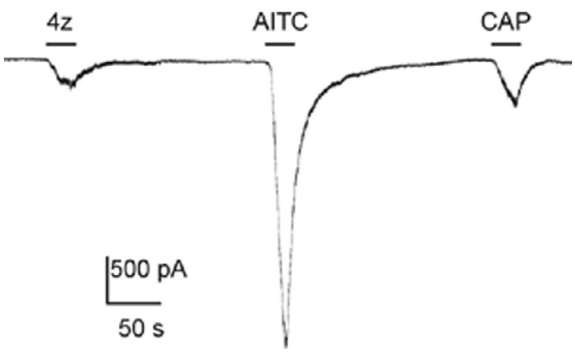


Fig. 4. HUHS029 (**4z**)-induced TRPA1 activation in DRG neurons. Representative trace showing **4z** (30 μM, 30 s)-, AITC (300 μM, 30 s) and capsaicin (CAP, 1 μM for 30 s)-induced currents in one DRG neuron.

behaviors. Consistent with our previous studies,[12] AITC injection without **4z** pretreatment induced a significant nocifensive behavior represented as increases in paw lifting and licking at the site of injection. Interestingly, injection of **4z** (2 mM, 30 μL in 10% DMSO in paraffin) itself did not cause acute nocifensive behavior such as paw lifting or licking. Paw-lifting is a reflex response occurs in the spinal level, while paw-licking is a conscious behavior which follows orders from the cerebral cortex. Therefore, licking time is thought to indicate pains of rats because they lift and lick paw when they feel pain. This lack of an irritant effect of **4z** may be due to its low potency and efficacy for activating TRPA1. As expected, the pretreatment with **4z** (5 min before AITC injection) resulted in a significant decrease in the licking time of the hind paw in the initial 5 min after the intraplantar AITC injection (Fig. 5).

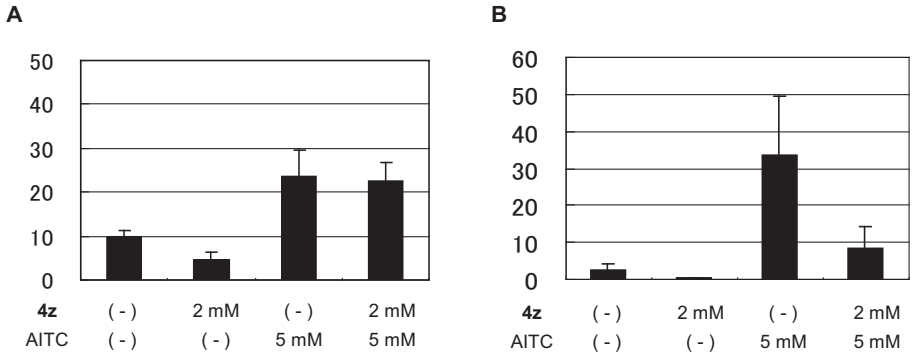


Fig. 5. Pretreatment with **4z** suppresses AITC-induced nocifensive pain behavior in rats. Five min before the AITC (5 mM, 30 uL) injection, **4z** (2 mM, 30 uL) or vehicle (10% DMSO in paraffin) were intraplantarly injected into the right hind paw (see method). Bar graphs showing the number of AITC-induced paw lifts (A) and the time of AITC-induced paw licking during the initial 5 min after AITC injection.

In conclusion, we designed and synthesized a series of resveratrol derivatives and tested their modulatory effect on the TRPA1 channel. Among the synthesized compounds, HUHS029 (**4z**) showed agonism and antagonism on the TRPA1 channel. Further electrophysiological and behavioral analysis indicated that **4z** can activate TRPA1 and subsequently desensitize the channel, and it therefore may serve as a candidate for novel analgesic targeting TRPA1 since HUHS029 (**4z**) is a derivative from the widely used natural product, resveratrol (**1**), and expected to be safety while other synthetic inhibitors arose from random screenings have been terminated by mainly side effects such as burning pain.

Acknowledgments

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A. Supplementary data

Supplementary data (experimental procedures and analytical data of compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.05.025.

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11. Typical procedure for the synthesis of resveratrol derivatives in this study (preparation of HUHS029 (**4z**)). To a solution of **7d** (400 mg, 1.3 mmol) in *p*-xylene (4 mL) was added **8k** (244 mg 1.5 mmol), *N*-ethylmorpholine (147 mg, 1.3 mmol) and then acetic acid palladium(II) salt (2.9 mg, 0.013 mmol) under nitrogen, which was stirred at 100 °C for 5 h. The reaction mixture was diluted with ethyl acetate and filtered with celite. The filtrate concentrated under reduced pressure. The resulting residue was purified using silica gel column chromatography (1% ethyl acetate in *n*-hexane) to give acetic acid 4-[2-(3,5-dibromo-4-methoxy-phenyl)-vinyl]-phenyl ester as a white powder (76 mg, 14%), was used for the next step without further purification. The intermediate (33 mg, 0.077 mmol) was diluted in dichloromethane (1.0 mL) and added 1.0 M dichloromethane solution of boron tribromide (0.23 mL, 0.23 mmol) under nitrogen and ice cooling. The mixture was stirred at room temperature for 2.5 h. The reaction mixture was added to cooled water under ice cooling and extracted with ethyl acetate. The combined organic layer was washed with water and brine, dried over anhydrous $mgSO_4$, filtered and then concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (20% ethyl acetate in n-hexane) to give **4z** as a white powder (26 mg, 91%). hTRPA1 screening assay was carried out as below: Calcium influx to the cells after stimulation of the receptor were examined using Calcium KitII-Fluo4 (DOJINDO, #CS32). The loading buffer of the kit was loaded to the hTRPA1-transfected HEK293 cells or untransfected cells in a 96-well plate, after 1 h, Ca^{2+}-dependent fluorescence intensity of each wells were previously measured for 30 sec with an excitation wavelength of 485 nm and an emission wavelength of 525 nm on FlexStation3 (Molecular Devices). Then, compounds or 0.1% DMSO in the culture medium were added and measured for 6 min at room temperature; after that, AITC or 0.1% DMSO was added, and immediately, the fluorescence intensity was measured for 1 min. The change value of fluorescence was calculated as the rate of the average fluorescence value, after compound administration/initial fluorescence average value for agonistic effect, after AITC administration/initial fluorescence average value for antagonistic effect, furthermore, inhibition (%) of compounds for AITC activation of hTRPA1 was also calculated about each compound as 0% inhibition at 0.1% DMSO control. To compare the inhibitory activity of the compounds, relative inhibitory activity of Resveratrol was calculated.
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