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Novel Non-vanilloid VR1 Antagonist of High Analgesic Effects and Its Structural Requirement for VR1 Antagonistic Effects

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Abstract—A novel non-vanilloid VR1 antagonist consisting of a new vanilloid equivalent exhibits excellent analgesic effects as well as highly potent antagonistic activities in both capsaicin single channel and calcium uptake assays. In addition, the structural requirement for the vanilloid equivalent of the potent VR1 antagonist has also been elucidated.

Introduction

Over the past few years, the amount of information from studies on pain transmission by capsaicin has dramatically increased, revealing novel targets for the advent of new pain therapies. Recently, a giant step forward came with the identification of a protein called the vanilloid receptor 1 (VR1). VR1 can be activated not only by vanilloid ligands including capsaicin and endocannabinoid, but also by noxious heat ($>43\,^{\circ}C$) and protons (extracelluar $pH<6$). Particularly, vanilloids and low pH are known to reduce the temperature threshold for VR1 activation.[1–3] Our recent studies have also revealed several 12-lipoxygenase metabolites of arachidonic acid, including 12-(*S*)-HPETE, as endogenous activators of the neuronal vanilloid receptor.[4]

Since capsaicin was found as an excellent vanilloid receptor agonist, considerable efforts toward the development of a novel analgesic have been continued. However, the small therapeutic window between these effects and the excitatory side effects, such as hyperthermia, bronchoconstriction, increased GI mobility, and hypertension, precluded the development of capsaicin as a systemic agent. Thus, recent studies on VR1 agonists or antagonists have focused on separating the excitatory effects of capsaicin analogues from the antinociceptive properties of these molecules.

In particular, the idea that VR1 functions as an integrator of multiple pain-producing stimuli implies that VR1 antagonists or channel blockers should have profound antinociceptive effects,[5] especially in inflammatory pain models.[6] Many research groups, including ours,[7] are engaged in developing potent and novel VR1 antagonists, although the therapeutically useful antagonists are not currently available.[2,8,9]

On the basis of the previous studies on vanilloid receptor agonists and antagonists, as well as our recent exciting findings,[4] we have looked for the non-vanilloid VR1 antagonists by developing the ideal vanilloid equivalents, which might provide the perfect analgesic effects without the side effects caused by vanilloid receptor agonists. Consequently, we have developed an excellent VR1 antagonist (SC0030) and revealed a part of the mechanistic aspect of SC0030.[10] We herein report the results of our recent studies on SC0030 from the view-

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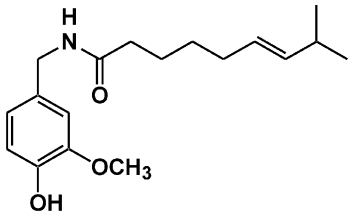
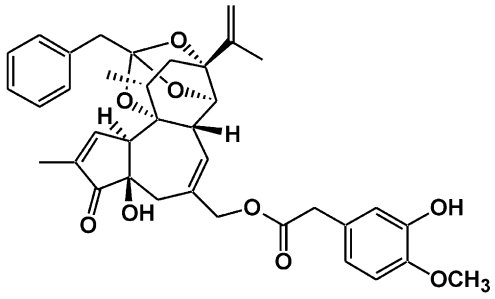


Capsaicin



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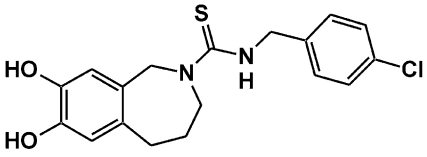


Capsazepine

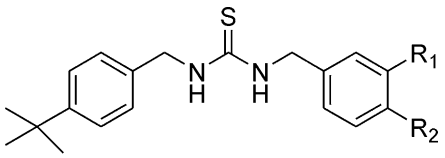
point of the minimal structural requirement for its potent VR1 antagonistic effects as well as its therapeutic application.

Since our initial work was focused on the development of novel vanilloid equivalents, which function as both hydrogen bonding donors and/or acceptors, like the vanilloid moiety of capsaicin, a large number of vanilloid equivalents have been designed and synthesized in our laboratory. The representative analogues, shown in Table 1, were selected for the comparison of their VR1 antagonistic effects because they have the characteristic functions of the vanilloid equivalent.

Chemistry

The advanced synthesis of SC0030[10] is outlined in Scheme 1. Cyanation of the methansulfonamide **2**, prepared by mesylation of the commercially available 2-fluoro-4-iodoaniline, with CuCN at 130 °C provided the cyano intermediate **3** in 75% of two steps yield. Conversion of the nitrile **3** to the benzylamine **4** was carried out by BH_3 reduction. Finally, the coupling of the amine salt **4** with 4-*t*-butylbenzyl isothiocyanate afforded the thiourea **5** (SC0030) in 75% of two steps yield.

Table 1. Ca^{2+} uptake inhibition of **SC0030** and its representative structural analogues in DRG neurons



Analogues	R_1	R_2	IC_{50} (μM)
5 (SC0030)	F	$NHSO_2CH_3$	0.037
10	$NHSO_2CH_3$	F	24.30
14 (MK056)	H	$NHSO_2CH_3$	0.110
17	F	H	>30
18	H	H	Not active
	Capsazepine		0.59

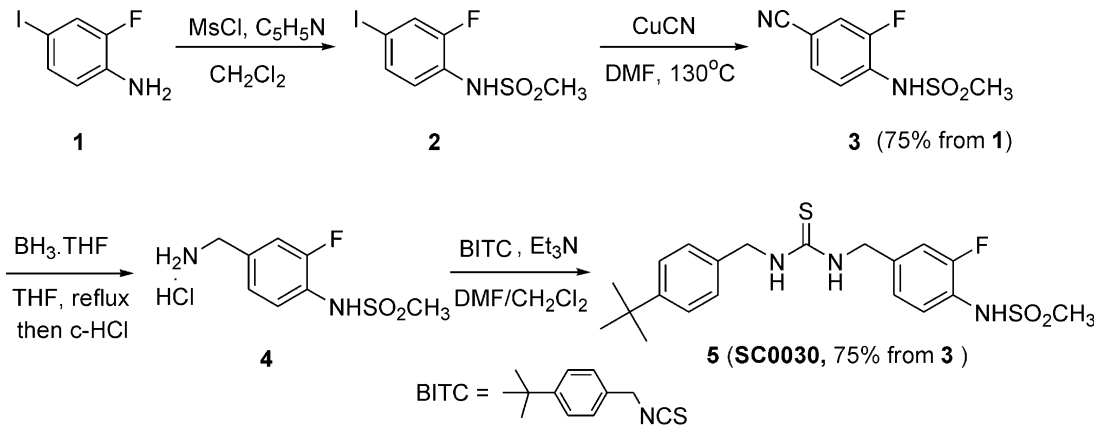
The analogue **10** was synthesized from the commercially available 4-fluoro-3-nitrobenzaldehyde **6**. Reaction of benzaldehyde **6** with hydroxylamine and reduction of the resulting oxime followed by Boc-protection provided the Boc-protected benzylamine **7**. Bismesylation of **7** and Boc-deprotection followed by coupling of the benzylamine salt with 4-*t*-butylbenzyl isothiocyanate afforded the thiourea **9** in 67% yield. Finally, selective removal of one mesyl group of **9** by NaOH treatment afforded the desired thiourea **10** in 54% yield (Scheme 2). The analogue **14** (MK056) was synthesized from *p*-nitrobenzylamine **11** by a five steps sequence. The sulfonamide intermediate **13** was prepared by Boc-protection of nitrobenzylamine **11**, catalytic reduction of nitro group, followed by mesylation of the resulting benzylamine. The sulfonamide **13** was converted to the analogue **14** by the procedure previously described[10] (Scheme 3). Analogues **17** and **18** were conveniently prepared by coupling of the corresponding benzylamine with 4-*t*-butylbenzyl isothiocyanate (Scheme 4).

Biological Assays

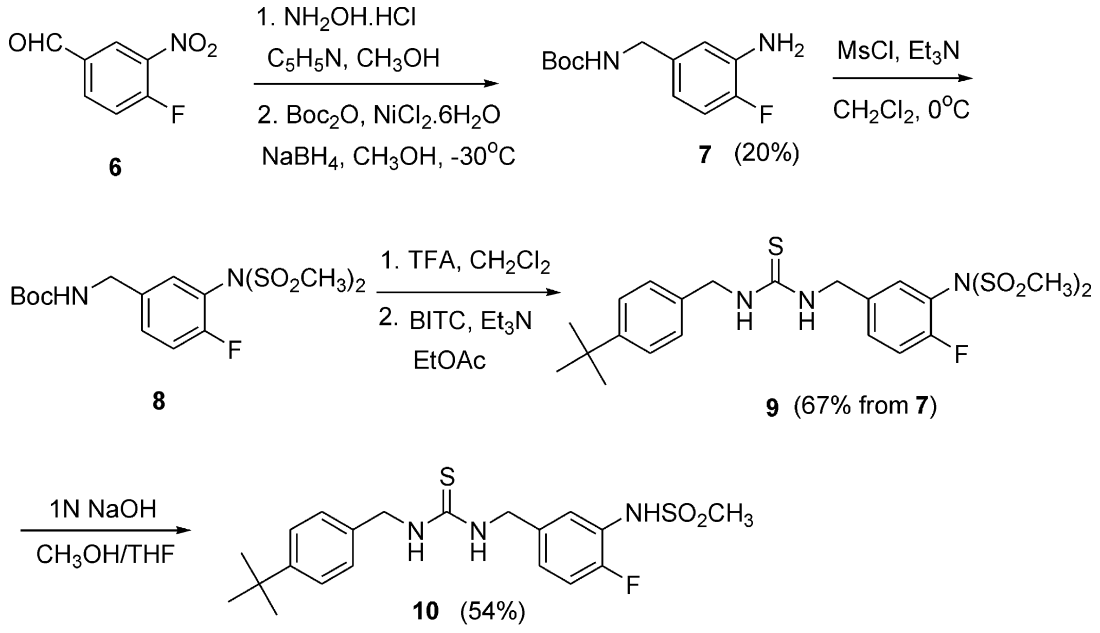
Dorsal root ganglion (DRG) neurons were prepared from neonatal Sprague–Dawley rats by a modification of the previously described method, and $^{45}Ca^{2+}$ uptake experiments were carried out by the reported procedure.[11] The IC_{50} is expressed as the concentration of the tested compound required to reduce the response to 0.5 μM capsaicin by 50%.

In order to determine whether the tested compounds activate or antagonize the vanilloid receptor, we recorded the single-channel current of the vanilloid receptor in inside-out membrane patches isolated from cultured sensory neurons.[4]

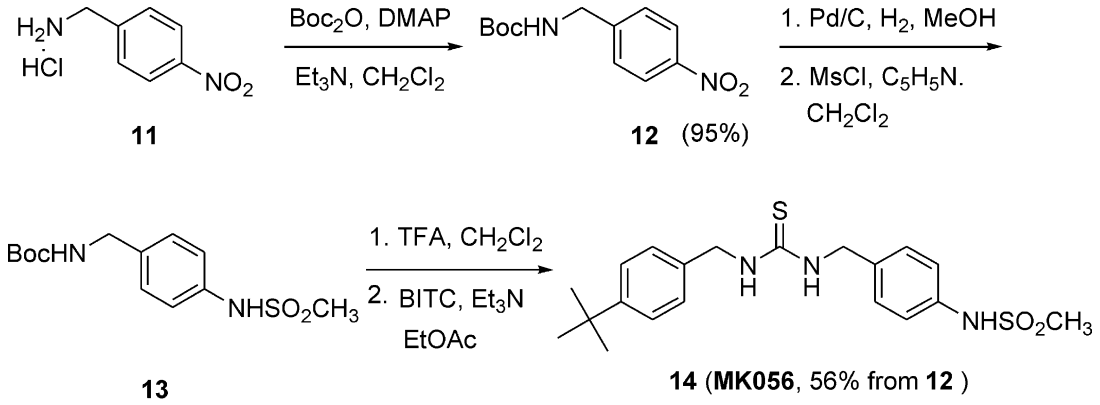
The PBQ-induced writhing assay carried out with male mice, which received an intraperitoneal injection of the chemical irritant phenyl-*p*-quinone.[12] A reduction in the number of writhes responding to phenyl-*p*-quinone relative to the number responding in the saline control



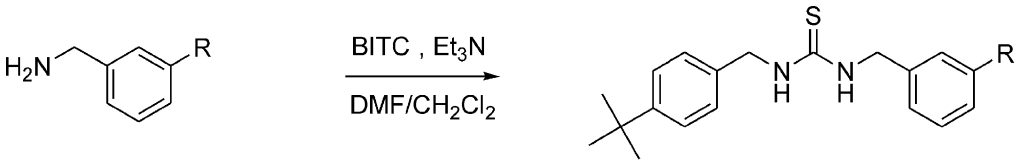
Scheme 1. Synthesis of analogue **5** (SC0030).



Scheme 2. Synthesis of analogue **10**.



Scheme 3. Synthesis of analogue **14** (MK056).



15 R = F

16 R = H

17 R = F (70%)

18 R = H (98%)

Scheme 4. Synthesis of analogues **17** and **18**.

group was considered to be indicative of an antinociceptive effect.

Results and Discussion

We initially examined the in vitro activities of more than hundred synthetic compounds, which were designed based on the structures of the reported natural and unnatural agonists and antagonists. The analogue SC0030 was finally selected as the best antagonist based on the in vitro assays employing calcium uptake of the vanilloid receptor. On the structural basis of the synthesized ligands,[13] the structural requirements of the vanilloid equivalents for the potent VR1 antagonistic effects were analyzed from Table 1.

Fluoride for R_1, which displaces methoxy of the vanilloid moiety of capsaicin turned out to be the best substituent as a H-bonding acceptor while methansufonamide for R_2, which displaces hydroxy of the vanilloid moiety was the best substituent as a H-bonding donor. In particular, SC0030, which possesses both fluoro and methansulfonamido substituents was 16-fold more potent than capsazepine in Ca^{2+} uptake inhibition, as a known VR1 antagonist. This novel ligand deserves great attention as a potent VR antagonist with an IC_{50} of 0.037 μM in Ca^{2+} uptake inhibition test, which makes it one of the most potent non-vanilloid VR antagonistic ligands described to date. The analogues possessing other substituents for R_1 and/or R_2 provided lower antagonistic effects or agonistic effects. Switch of fluoro (R_1) and methansulfonamido (R_2) groups significantly drops Ca^{2+} uptake inhibition activity. Elimination of fluoro group for R_1 (**14**) decreases the antagonistic activity. However, the analogue **14** still exhibits the higher potency than capsazepine. The analogue **17**, which possesses only fluoro substituent for R_1 retains antagonistic activity although the potency is quite low. However, the analogue **18**, which possesses neither fluoro nor methansulfonamido substituent, completely loses the antagonistic activity. In conclusion, the fluoro and methansulfonamido groups for R_1 and R_2, respectively, are obviously essential for the high Ca^{2+} uptake inhibition activity of the vanilloid equivalent. The fluoro substituent itself provides weak antagonistic activity. However, it is crucial for the high antagonistic activity of the vanilloid equivalents.

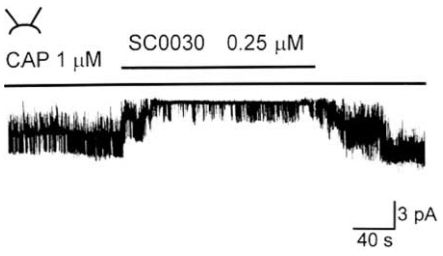


Figure 1. Reversible antagonistic effects of SC0030 on the capsaicin receptor.

Consistent with observation of calcium uptake results, the VR1 antagonism of SC0030 was confirmed by inhibition of the capsaicin-induced action on patch-clamped rat DRG neurons. As shown in Figure 1, the application of 1 μM of capsaicin greatly activates capsaicin receptors in inside-out membrane patches. However, SC0030 inhibited the channel activity (85.7±2.4% reduction, $n=4$) evoked by capsaicin when 0.25 μM of SC0030 was applied to the patch together with 1 μM of capsaicin. After the SC0030 was removed, the application of 1 μM of capsaicin again activated the channel activity. Thus, these results suggest that 0.25 μM SC0030 clearly antagonizes the action of capsaicin at the capsaicin receptor in a reversible manner. Figure 2 shows the magnitude of inhibition by 0.25 μM of SC0030 was comparable to the magnitude obtained after the application of 10 μM of capsazepine.

The analgesic effect of SC0030 was evaluated from the viewpoint of the therapeutic applications and it was confirmed by the PBQ-induced writhing antinociceptive assay. The results presented in Figure 3 show that

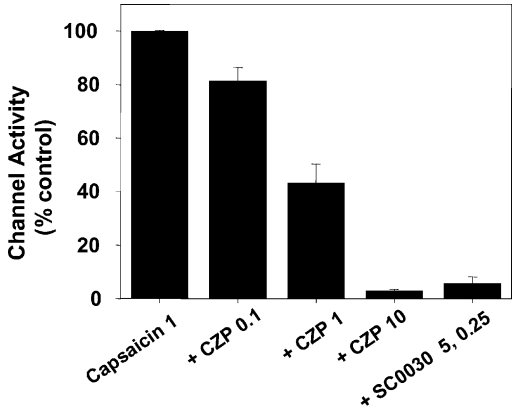


Figure 2. Comparison of the antagonistic effects of SC0030 and capsazepine on capsaicin receptor activity in sensory neurons. Numbers represent concentrations in μM.

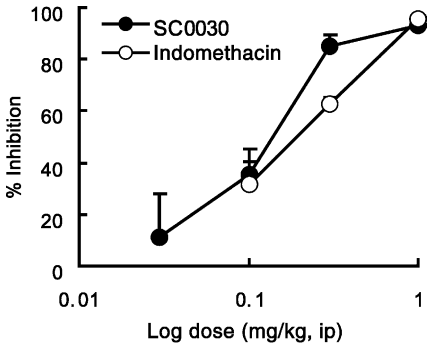


Figure 3. Potent and dose-dependent analgesic effect of SC0030 on PBQ-induced writhing in mice ($n=10$/group). Values (mean ± SEM) represent % inhibition of writhing responses.

SC0030 inhibited the writhing in a dose-dependent manner, and was almost equipotent or slightly more potent than indomethacin, a nonselective COX inhibitor.

For further confirmation of the therapeutic uses of SC0030, we examined the tests related to the pungency, which generally arises from the agonistic effects of capsaicin analogues. We performed a capsaicin-induced licking test,[14] on the basis that the capsaicin-evoked activation of sensory afferent neuron is associated with the activation of selective membrane vanilloid receptors. SC0030 dose-dependently inhibited capsaicin-induced nociception as well as pain-related behavioral responses. In addition, SC0030 did not cause the protective eye-wiping movement in the rat upon intraocular instillation. The topical application of the capsaicin to the eye of experimental animals is known to evoke immediate pain, as revealed by the increased number of scratching movements toward the treated eye.

As mentioned earlier, the previous studies on VR1 agonists reported that systemic and intrahypothalamic injection of capsaicin produce hypothermia. The VR1 expressing neurons in the brain area are thought to play an important role in the central control of thermoregulation. As we anticipated, no change of body temperature by the administration of SC0030 in the rat was observed.

In summary, in the present work we have elucidated the structural requirement for the potent and selective VR1 antagonist, particularly for the vanilloid equivalent. The synthetic SC0030 acts as a strong inhibitor of Ca^{2+} uptake, with a much lower IC_{50} value than that of capsazepine. In addition, it displays a potent analgesic activity similar to that of indomethacin in vivo. Moreover, this compound is devoid of the important shortcomings of capsaicin, such as hypothermia and pungency. Most importantly, SC0030 can be conveniently synthesized on more than a hundred-gram scale to ensure a substantial quantity. This novel VR1 antagonist would be highly useful not only as a new candidate for non-vanilloid analgesic development but also as a tool to investigate the VR1-mediated pain response.

Acknowledgements

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