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Letter

Identification and Characterization of Novel Bronchodilator Agonists Acting at Human Airway Smooth Muscle Cell TAS2R5

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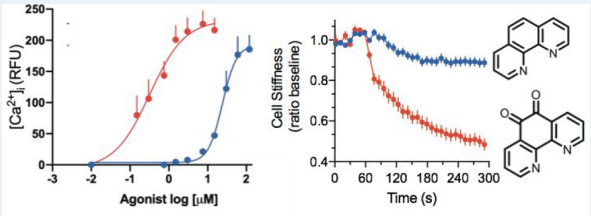
ABSTRACT: Bitter taste receptors (TAS2Rs) are recognized as being expressed on multiple cell types and organs, including human airway smooth muscle (HASM) cells, where agonists promote significant relaxation to constrictive stimuli. Thus, the HASM TAS2Rs have been targeted as novel bronchodilators for the treatment of asthma and other obstructive lung diseases. The TAS2R5 subtype, a dominant receptor on HASM, has few known agonists, all with reported low potency and efficacy. We screened multiple compounds by measuring $[Ca^{2+}]_i$ release in HASM (a consequence of receptor−G protein coupling) to establish structure−activity relationships and arrive at a potent agonist for TAS2R5. HASM physiological studies using magnetic twisting cytometry confirmed the relaxation effects of lead compounds. 1,10-Phenanthroline-5,6-dione had the greatest potency ($EC_{50} \approx 120$ nM), amounting to a >1000-fold improvement over the other compounds, and displayed maximal efficacy. These studies revealed critical structural requirements for favorable potencies and efficacies for a potential first-in-class bronchodilator targeting TAS2R5 of the airway.



KEYWORDS: *bitter taste receptors, G protein-coupled receptors, airway smooth muscle, magnetic twisting cytometry, Ca^{2+}*

Bitter taste receptors (TAS2Rs) are members of the G protein-coupled receptor (GPCR) superfamily of receptors and traditionally were thought to have expression confined to taste cells,[1] where they detect bitter substances. The human genome encodes 25 TAS2R subtypes with varying degrees of specificity to bitter tastants.[2] Some subtypes are described as being "broadly tuned", while others appear to have highly restrictive requirements for agonist binding and activation.[3] Recent studies have shown that TAS2Rs are expressed on multiple cell types and tissues throughout the body,[4,5] representing a previously unrecognized chemosensory system responding to endogenous and exogenous substances, including potential novel therapeutic targets.

Of particular interest has been the delineation of six TAS2R subtypes (TAS2Rs 4, 5, 10, 14, 19, and 31) that are expressed on human airway smooth muscle (HASM) cells.[6] TAS2R activation in these cells results in coupling of the receptor to the Gαi family of G proteins[7] with a subsequent increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in microdomains that leads to marked HASM relaxation (Figure 1a).[6] This has prompted investigations into the use of TAS2R agonists as a potential treatment for obstructive lung diseases such as asthma, where HASM contraction is a main mechanism of airflow restriction.[8,9] Five of the aforementioned six TAS2R subtypes appear to be broadly tuned, being activated by compounds with wide-ranging structural properties, albeit with typically low apparent affinities.[2] These properties may be due to the evolution of TAS2Rs on taste cells in order to detect the large number of bitter-tasting toxic substances found in plants.[2] This diversity is illustrated by studies with TAS2R14, which show that it is activated by quinine, aristolochic acid, chlorhexidine, and flufenamic acid as well as >29 other known compounds.[2] Furthermore, many of these agonists also activate other TAS2R subtypes that are not expressed on HASM but are found in other organs such as heart, thyroid, pancreas, and uterus.[4,5] For drug development, this lack of more specific high-affinity binding requirements of TAS2R binding pockets for activation represents a challenge for identifying receptor ligands for therapeutic purposes.

Currently, the only available direct bronchodilators are β_2-adrenergic receptor (β_2AR) agonists, also called "β-agonists". These agents activate the cell surface β_2AR on HASM and activate Gαs, stimulating adenylyl cyclase and increasing cellular cAMP and activation of protein kinase A, which leads to relaxation. β-Agonists are associated with a number of adverse effects, and about half of asthmatics fail to reach optimal control.[10] Thus, efforts to find new classes of direct bronchodilators targeting a number of HASM proteins are underway.[11] These include not only TAS2Rs but also Rho kinase inhibitors, prostanoid receptor agonists, peroxisome

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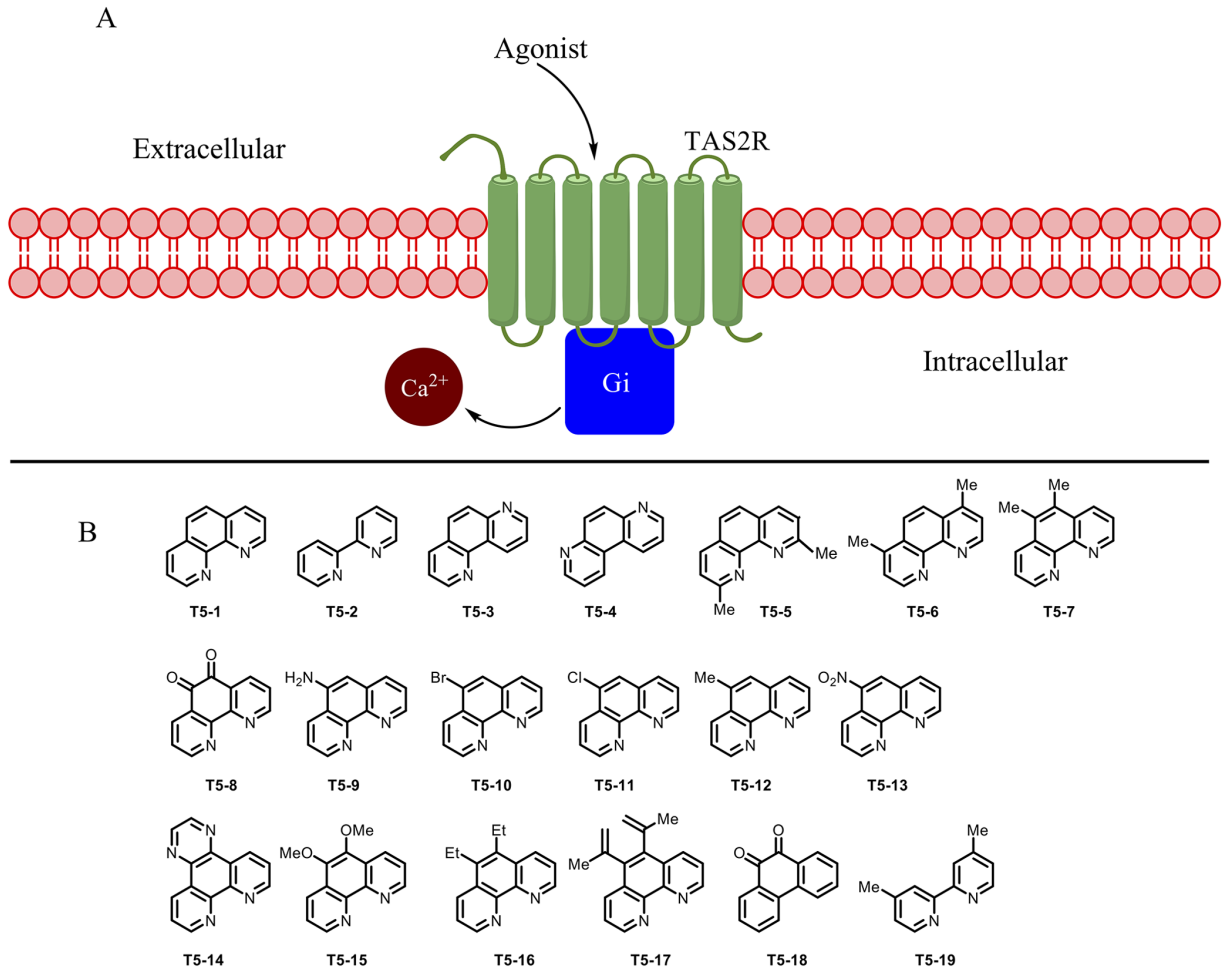

Figure 1. TAS2R agonist signaling and the structures of potential TAS2R5 agonists that were screened. (a) General signaling paradigm of TAS2Rs in airway smooth muscle cells. The agonist binds to residues in a pocket formed by the hydrophobic transmembrane domains of the receptor, resulting in a conformation that binds and activates the heterotrimeric G protein G_i, ultimately causing an increase in $[Ca^{2+}]_i$. (b) Structures of the compounds screened for $[Ca^{2+}]_i$ stimulation and relaxation in HASM cells.

proliferator-activated receptor γ agonists, and pepducins that modify GPCR signaling.[11] Our studies of HASM TAS2Rs for potential new bronchodilator targets are based on experiments where we find a high level of efficacy for relaxation of human bronchi,[8] no effect of the asthma cellular phenotype on TAS2R function,[12] additive effects with β-agonists[6] without cross-desensitization,[13] and their distinct mechanism of action.[6]

In the most extensive screening to date,[2] only one agonist, 1,10-phenanthroline (here termed **T5-1**; Figure 1b) was found for TAS2R5 out of the >80 bitter compounds studied, and this compound did not activate the other human TAS2Rs, including those with closely related transmembrane domain sequences. The potency from this screen was not reported, but the minimal concentration that elicited an increase in $[Ca^{2+}]_i$ in cells was noted to be 100 μM, indicating an apparent low-affinity interaction. Several natural substances may also activate TAS2R5 (see Figure S-1), but the potencies and/or maximal responses appear to be low.[14] In the current work, we explored agonist activation of TAS2R5 in cell- and physiological-based systems using as a starting point the structure of **T5-1** as a verified agonist. Our goal was an improvement in potency and/or efficacy in order to define structure−activity relationships for this receptor, opening the potential for agonists for this target to represent first-in-class bronchodilators for treating asthma.

Cultured D9 telomerase reverse transcriptase immortalized HASM (D9-HASM) cells were utilized for the $[Ca^{2+}]_i$ screening assays.[15] Cells were seeded onto 96-well plates and loaded with Fluo-4 direct. The $[Ca^{2+}]_i$ responses to multiple concentrations of potential agonists were determined as a measure of receptor activation. Control cells were exposed to the nonspecific ionophore ionomycin at 2.0 μM (the maximal effective concentration; Figure S-2). Results from TAS2R agonist studies were normalized to the ionomycin response in order to compare the maximal responses (R_{max}) between agonists. The concentration−response data were fit to a four-component logistic regression model (sigmoid curve) using iterative nonlinear regression methods to determine the values of EC_{50} and R_{max}.[16] The structures of the previously identified agonist **T5-1** and 18 derivatives (denoted as **T5-2** through **T5-19**) are shown in Figure 1b. Representative $[Ca^{2+}]_i$ responses to these compounds are shown in Figure S-2, and mean results

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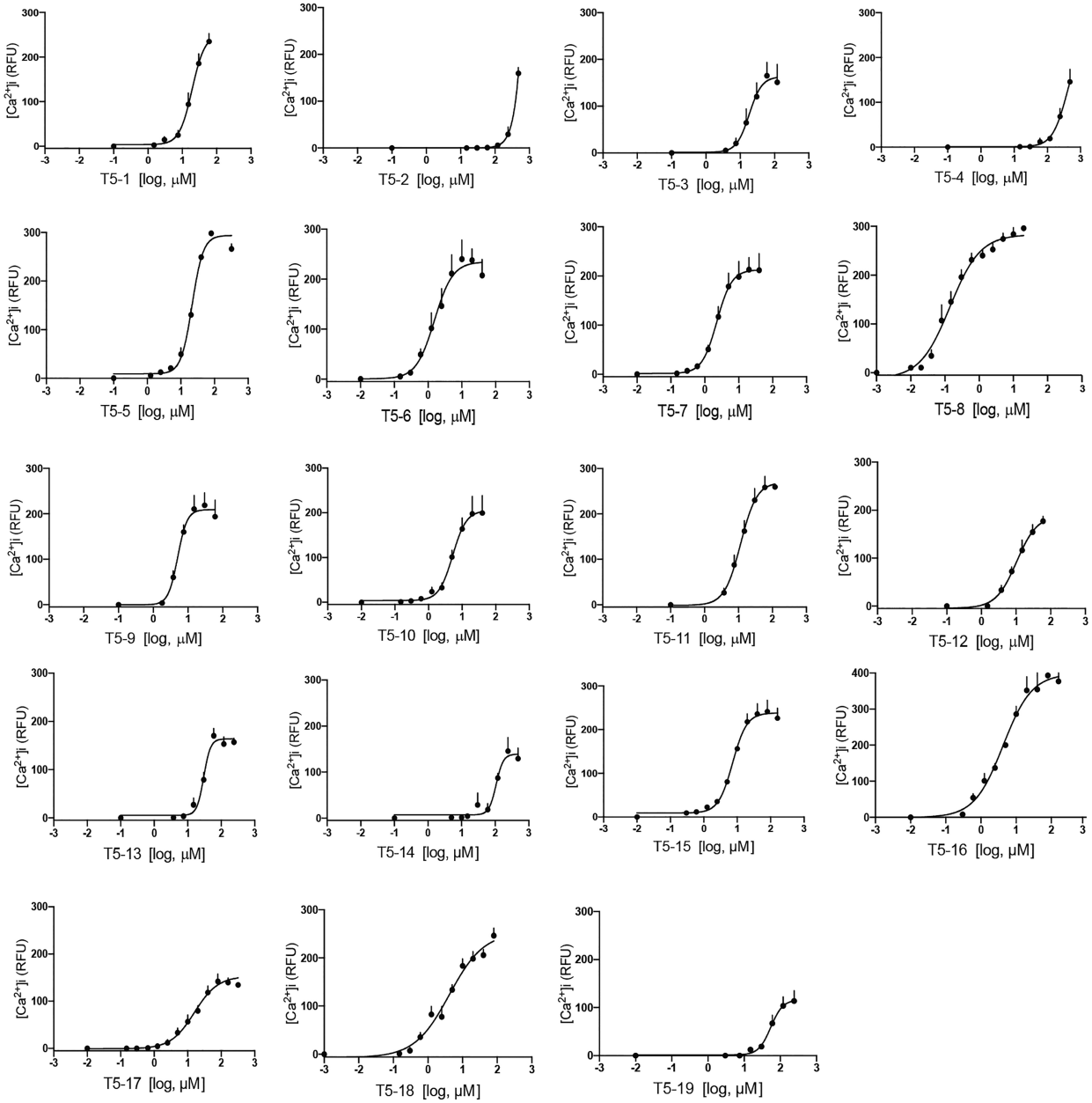

Figure 2. Concentration−response curves of compounds screened for stimulation of $[Ca^{2+}]_i$ in D9-HASM cells. Results are reported as mean ± SE from four to eight experiments. See Table 1 for EC_{50}, R_{max}, and statistical comparisons.

from multiple concentration−response experiments are shown in Figure 2. Table 1 summarizes the results in terms of potency (EC_{50}) and efficacy (R_{max}), with statistical comparisons to **T5-1**.

The parent compound **T5-1** revealed an EC_{50} of ∼30 μM with a maximal response of ∼149% of ionomycin (Table 1). The flat tricyclic ring structure appears to be required, as the response of the corresponding bipyridine analogue **T5-2** was low (16% of ionomycin) and could not be resolved as a sigmoid curve. Some of the activity could be restored via substitution (**T5-19**), suggesting that additional hydrophobicity away from the nitrogens may be required. The relatively equal activities of **T5-1** and **T5-3** demonstrate that only one nitrogen is required on the concave face of this scaffold. The inactivity of **T5-4**, however, shows that at least one of these nitrogens must be present on the concave face (Figure 1b and Table 1).

Additional substitutions around the **T5-1** structure proved to be quite revealing. Methyl groups that extended into the concave portion of the molecule had little effect (**T5-5**), which implies that the nitrogens are not sensitive to steric bulk. Substitution on the convex face of the molecule had variable effects on the responsiveness, sometimes resulting in a marked improvement in both the EC_{50} and the R_{max} (**T5-6** through **T5-17**). This improvement seemed to be largely independent of hydrogen-bond-donating (e.g., **T5-9**) or -accepting (e.g.,

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Table 1. $[Ca^{2+}]_i$ Stimulation and Smooth Muscle Relaxation Responses of HASM Cells to the Indicated Compounds

agonist	abbreviation	EC_{50} (μM)	R_{max} (% of ionomycin)	relaxation (% of basal)
1,10-phenanthroline	**T5-1**	29.7 ± 5.57	149 ± 10.23	10.8 ± 2.17
2,2′-bipyridine	**T5-2**	N/A[c]	16.4 ± 4.48	−3.2 ± 1.67
1,7-phenanthroline	**T5-3**	24.9 ± 4.58	121 ± 16.7	34.8 ± 1.36[b]
4,7-phenanthroline	**T5-4**	N/A	15.4 ± 3.37[a]	15.3 ± 1.48
2,9-dimethyl-1,10-phenanthroline	**T5-5**	33.3 ± 6.40	143 ± 13.1	12.0 ± 1.47
4,7-dimethyl-1,10-phenanthroline	**T5-6**	1.39 ± 0.19[a]	147 ± 16.7	5.3 ± 1.95
5,6-dimethyl-1,10-phenanthroline	**T5-7**	2.05 ± 0.20[a]	163 ± 36.4	9.0 ± 2.19
1,10-phenanthroline-5,6-dione	**T5-8**	0.12 ± 0.05	199 ± 20.2[a]	50.2 ± 2.79[b]
1,10-phenanthrolin-5-amine	**T5-9**	4.60 ± 0.42[a]	176 ± 14.2	7.2 ± 1.95
5-bromo-1,10-phenanthroline	**T5-10**	4.35 ± 0.71[a]	182 ± 25.7	14.4 ± 1.93
5-chloro-1,10-phenanthroline	**T5-11**	11.1 ± 2.00	183 ± 7.3	18.0 ± 2.13[b]
5-methyl-1,10-phenanthroline	**T5-12**	12.1 ± 1.99	181 ± 24.6	ND[d]
5-nitro-1,10-phenanthroline	**T5-13**	36.0 ± 3.85	134 ± 11.9	12.8 ± 2.00
pyrazino[2,3-*f*] [1,10]phenanthroline	**T5-14**	146.6 ± 2.26[a]	34.5 ± 15.1[a]	6.1 ± 1.67
5,6-dimethoxy-1,10-phenanthroline	**T5-15**	7.48 ± 0.78[a]	127 ± 16.1	26.7 ± 1.65[b]
5,6-diethyl-1,10-phenanthroline	**T5-16**	4.05 ± 0.80[a]	183 ± 14.7	ND
5,6-bis(prop-1-en-2yl)-1,10-phenanthroline	**T5-17**	25.5 ± 7.06	113 ± 19.0	13.5 ± 2.59
9,10-phenanthrenequinone	**T5-18**	5.08 ± 1.37[a]	153 ± 5.2	ND
4,4′-dimethyl-2,2′-bipyridine	**T5-19**	60.6 ± 9.13[a]	58.9 ± 2.95[a]	ND

[a]EC_{50} or R_{max} different from that of **T5-1**, $P < 0.05$ ($[Ca^{2+}]_i$ assays). [b]Relaxation greater than that of **T5-1**, $P < 0.01$ (MTC assays). [c]Not applicable because of lack of curve fit. [d]The experiment was not done.

T5-15) characteristics, though the strongly electron-withdrawing nitro substituent (**T5-13**) was demonstrably less active. As the substituents became considerably larger (e.g., **T5-14** and **T5-17**), the activity also appeared to diminish.

Unquestionably, the most potent agonist was **T5-8**, which is an *o*-quinone analogue of **T5-1** (Figure 1b). For **T5-8**, the potency was increased by ∼250-fold (EC_{50} = 0.120 μM vs 29.7 μM) while the R_{max} was maintained compared to **T5-1** (Figure 2 and Table 1). Alternative substitutions at the 5,6 position(s) with methyl, ethyl, Br, or Cl (**T5-7**, **T5-16**, **T5-10** and **T5-11**, respectively) improved the potency somewhat compared with **T5-1**, but this never reached the submicromolar potency of **T5-8**, pointing toward the importance of the *o*-quinone functionality. In addition, **T5-18**, which lacks both of the 1,10 nitrogens of **T5-8** and other agonists (Figure 1b), showed improved potency compared with **T5-1** but was still ∼50-fold less active than **T5-8**, further indicating that this *o*-quinone functionality imparts activity. The $[Ca^{2+}]_i$ responses to **T5-1** and **T5-8** were also determined in cultured primary HASM cells derived from a nonasthmatic lung. The potencies were similar for these two compounds using the primary cell line compared to the immortalized line (Figure S-3).

To correlate the $[Ca^{2+}]_i$ responses of the compounds to relevant physiological function, we turned to measurements of single-cell mechanics in HASM cells using magnetic twisting cytometry (MTC) as previously described.[6] Here, cultured primary HASM cells at passages 5−7 derived from a nonasthmatic donor lung were utilized. The decrease in cell stiffness evoked by a compound on the ferrimagnetically tagged HASM cells twisted by a magnetic field has been found to correlate with clinical airway relaxation.[17] For these studies, a single concentration of all agonists was utilized, recognizing that the 3 log differences in EC_{50} values observed with the compounds would yield a range of relaxation responses consistent with the structure−activity relationships established in the $[Ca^{2+}]_i$ assays, with some caveats (see below). Positive controls (Figure S-4) included the β-agonists albuterol (partial agonist), formoterol (full agonist), and forskolin, which increases cAMP by direct activation of adenylyl cyclase. The results from these studies are shown in Figure 3 and Table 1.

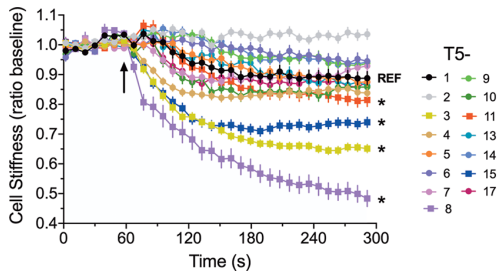

Figure 3. HASM cell relaxation responses to selected compounds. Compounds were studied at a concentration of 500 μM and were added to the culture medium at the 60 s time point (arrow). Results are from measurements of 200−400 cells per compound. *, $P < 0.01$ vs the **T5-1** reference response.

The reference compound **T5-1** achieved a modest degree of relaxation amounting to ∼11%, while **T5-8** evoked ∼50% relaxation, confirming the key findings of the structure−activity relationships derived from the $[Ca^{2+}]_i$ assays, where **T5-8** was the most potent agonist. The virtually inactive compound **T5-2** caused no detectable relaxation. A number of compounds were clustered between 5 and 15% relaxation, even though some of them differed in potency in the $[Ca^{2+}]_i$ assays, suggesting a threshold effect for this physiological response as well as a system with less sensitivity and intrinsically more noise. **T5-15** evoked an intermediate relaxation response consistent with the potency in the $[Ca^{2+}]_i$ assays that was higher than that of **T5-1** but lower than that of **T5-8**. An intermediate response, also in concordance with the $[Ca^{2+}]_i$ data, was observed with the relaxation to **T5-11** (Figure 3). A discrepancy between the $[Ca^{2+}]_i$ assay and the relaxation results was found with **T5-3**, which caused relaxation greater than that of the parent compound **T5-1** but had EC_{50} and R_{max} values in the $[Ca^{2+}]_i$ assays that were comparable to those of **T5-1**. **T5-4** exhibited

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some degree of relaxation, but the dose−response in the $[Ca^{2+}]_i$ assays could not be fit to a sigmoid curve, suggesting an atypical mechanism of action. These results may indicate off-target effects with **T5-3** and **T5-4**. The maximal relaxation response to **T5-8** was substantially greater than that of albuterol (50% vs 27%) and slightly greater than that of formoterol (44%) (see Figures 3 and S-4).

The only class of direct bronchodilators for treating airway obstruction are β-agonists.[11] While these agents are utilized for both acute treatment and prevention of bronchospasm in asthma, they have been variably reported to exhibit a number of undesirable characteristics. These include tachyphylaxis (loss of effectiveness with regular use),[18,19] decreased efficacy evoked by asthma pathobiology,[6,20,21] increases in airway contractile hyperresponsiveness,[22,23] loss of the bronchoprotective effect to constrictive stimuli,[24,25] interindividual variability from common receptor polymorphisms,[26−28] and an increase in exacerbations and mortality.[29−35] Preclinical studies to date indicate that agonists to TAS2RS expressed on HASM bronchodilate by a different mechanism[6] than β-agonists ($[Ca^{2+}]_i$ vs cAMP), are more efficacious,[6,8] display less tachyphylaxis,[36] are unaffected by crosstalk with asthmatic-associated signaling,[12] and particularly for TAS2R5 are less affected by genetic variation.[37] The different mode of action and the lack of some of the deleterious properties of β-agonists suggest a new target for a different class of bronchodilators. A previous extensive screen of bitter-tasting compounds showed that none of the compounds that activated the other human TAS2Rs activated TAS2R5 and that the one TAS2R5 agonist found from the screen (**T5-1**) failed to activate the other TAS2Rs.[2] This suggests that TAS2R5 may have an atypical or restrictive agonist binding pocket for activation compared with most of the other TAS2Rs. Thus, TAS2R5-targeted agonists may display efficacy in the lung for asthma treatment while having fewer adverse effects (particularly when inhaled) due to activation of other nongustatory TAS2Rs subtypes.

On the basis of the physiochemical properties of **T5-8**, this compound is well within the parameters for a high-potency lead in a discovery effort for TAS2R5 agonists. Specifically, it passes Ro4 criteria,[38] with a molecular weight of 210 g/mol, no H-bond donors, four H-bond acceptors, a cLogP of 0.66, and a total polar surface area (tPSA) of 59. Phenanthroline derivatives can have coordinating functions, primarily through chelation, that can facilitate actions when complexed with other drugs[39] or by acting alone, as illustrated by inhibition of matrix metalloproteinase.[40] We recognize that phenanthroline derivatives might act via chelation or other mechanisms to interfere with assays. We contend that this is not likely in the current work for several reasons. First, we utilized two very different assays to ascertain the activity: a biochemical assay and a physiological assay. The main findings were the same in both the $[Ca^{2+}]_i$ studies and the HASM cell relaxation studies, which argues against assay interference as the basis for the observations. Second, movement of one nitrogen from the concave face to the convex face (**T5-1** vs **T5-3**), resulted in no loss of activity, yet the chelation potential would be expected to be decreased for **T5-3**. Finally, **T5-1** has been reported to stimulate $[Ca^{2+}]_i$ only from activation of the TAS2R5 subtype, while the other human TAS2Rs that also couple to $[Ca^{2+}]_i$ stimulation and were assessed in the same assay showed no activity from **T5-1**,[2] which would be unexpected if assay interference were in play.

No crystal structure of any TAS2R has been delineated to date, and the amino acid homology of these receptors compared to GPCRs with known X-ray crystal structures is low (<20%). Thus, we cannot predict with any degree of certainty the agonist binding pocket of TAS2R5 or the structural basis for the apparent high affinity of agonists such as **T5-8** compared with the other compounds studied. Nevertheless, we have successfully defined critical components of a novel agonist with an EC_{50} of ∼120 nM. This potency is similar to those of some β-agonist bronchodilators currently available by inhalation, including albuterol, terbutaline, metaproterenol, and salmeterol.[41−45] For example, albuterol, which has a higher EC_{50} than **T5-8**, is administered at a dose of 2.5 mg in 3 mL of saline (3.4 mM) over 5 min by a hand-held nebulizer. Therefore, on the basis of its solubility and potency, **T5-8** is feasible for administration by this route, where the particle size from the device determines the optimal regions of deposition, which for bronchodilators are the conducting airways.[46]

While the current work has identified key structural requirements for high-potency TAS2R5 agonists, we are not in a position to assume that **T5-8** (or similar compounds that we identified) is necessarily the optimal TAS2R5 agonist for treating asthma. Additional preclinical studies, including pharmacodynamics and pharmacokinetics, toxicology, and adverse effect profile, will further guide additional modifications as indicated. Nevertheless, the structure−activity relationships that we have established form the basis for further development and indicate that it is possible to achieve high-potency activation of this receptor.

MATERIALS AND METHODS

Cell Culture. Primary cultured HASM cells were derived as described previously[6] from nonasthmatic donor lungs obtained from the National Disease Research Interchange (http://ndriresource.org) and used at passages 5−7. Cells were grown in Dulbecco's modified Eagle's medium and HAM's F12 medium (1:1) with 10% fetal calf serum and 1% penicillin and streptomycin in a 5% CO_2, 95% air atmosphere at 37 °C. The immortalized D9-HASM cell line was utilized as previously described[7] and maintained in Dulbecco's modified Eagle's medium with 10% bovine calf serum and 1% penicillin and streptomycin in the aforementioned atmosphere.

$[Ca^{2+}]_i$ Stimulation. Cultured D9-HASM or primary HASM cells were plated at 20 000 cells/well in 96-well plates and the next day loaded with Fluo-4 direct (Life Technologies), and $[Ca^{2+}]_i$ was detected using FlexStation 3 as described previously.[6] Cells were treated with vehicle, the indicated concentrations of compound, or 2.0 μM ionomycin, which served as a control for normalization of the maximal response. Compounds that were tested (>95% purity) were either commercially available or were synthesized by WuXi AppTec (Wuhan, China).

Magnetic Twisting Cytometry. MTC studies were performed as previously described in detail.[6] Briefly, integrin receptors on the surface of primary HASM cells were ligated to arginylglycylaspartic acid-coated ferrimagnetic microbeads and then subjected to magnetic fields to quantitate mechanical responses. Cells were magnetized horizontally and then twisted in a vertically aligned magnetic field. A decrease in stiffness (relaxation) was quantified by lateral bead displacement in response to the application of various compounds to the medium. After the baseline stiffness was established for 60 s,

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the compounds were then added to the medium, and the response was observed over the next 220 s.

Statistical Analysis. Concentration−response curves were generated by fitting the $[Ca^{2+}]_i$ data using iterative nonlinear four-component logistic regression with Prism 8.0 using eq 1:

$$Y = R_{min} + \left[\frac{R_{max} - R_{min}}{1 + \left(\frac{10^{\log EC_{50}}}{10^{X}} \right)^{n_H}} \right] \quad (1)$$

where Y is the response at a given concentration of compound, R_{min} and R_{max} are the minimal and maximal responses, respectively, X is the log of the concentration of the compound, EC_{50} is the concentration that evokes 50% of the response, and n_H is the Hill slope.[47] The results were compared using paired or unpaired t tests with significance imparted when $P < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.0c00127.

Structures of TAS2R5 agonists and albuterol (Figure S-1), representative $[Ca^{2+}]_i$ responses in D9-HASM cells (Figure S-2), $[Ca^{2+}]_i$ responses to **T5-1** and **T5-8** in primary HASM cells (Figure S-3), and HASM relaxation responses to albuterol, formoterol, and forskolin (Figure S-4) (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting information

Identification and characterization of novel bronchodilator agonists acting at human airway smooth muscle cell TAS2R5

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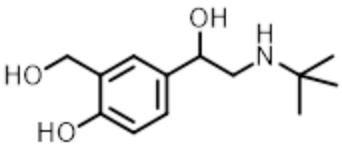
Contents:

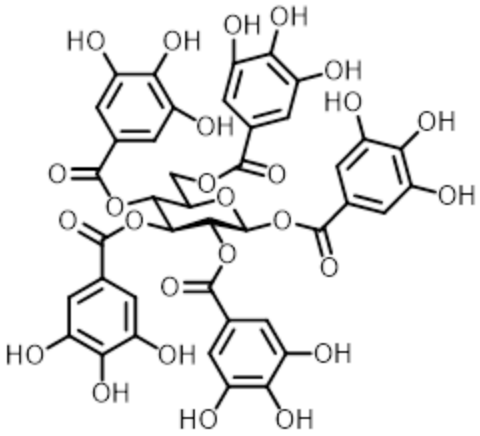
Figure S-1

A



albuterol

B



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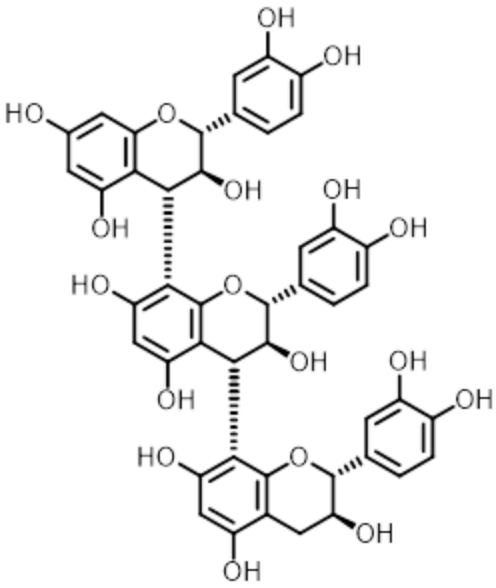
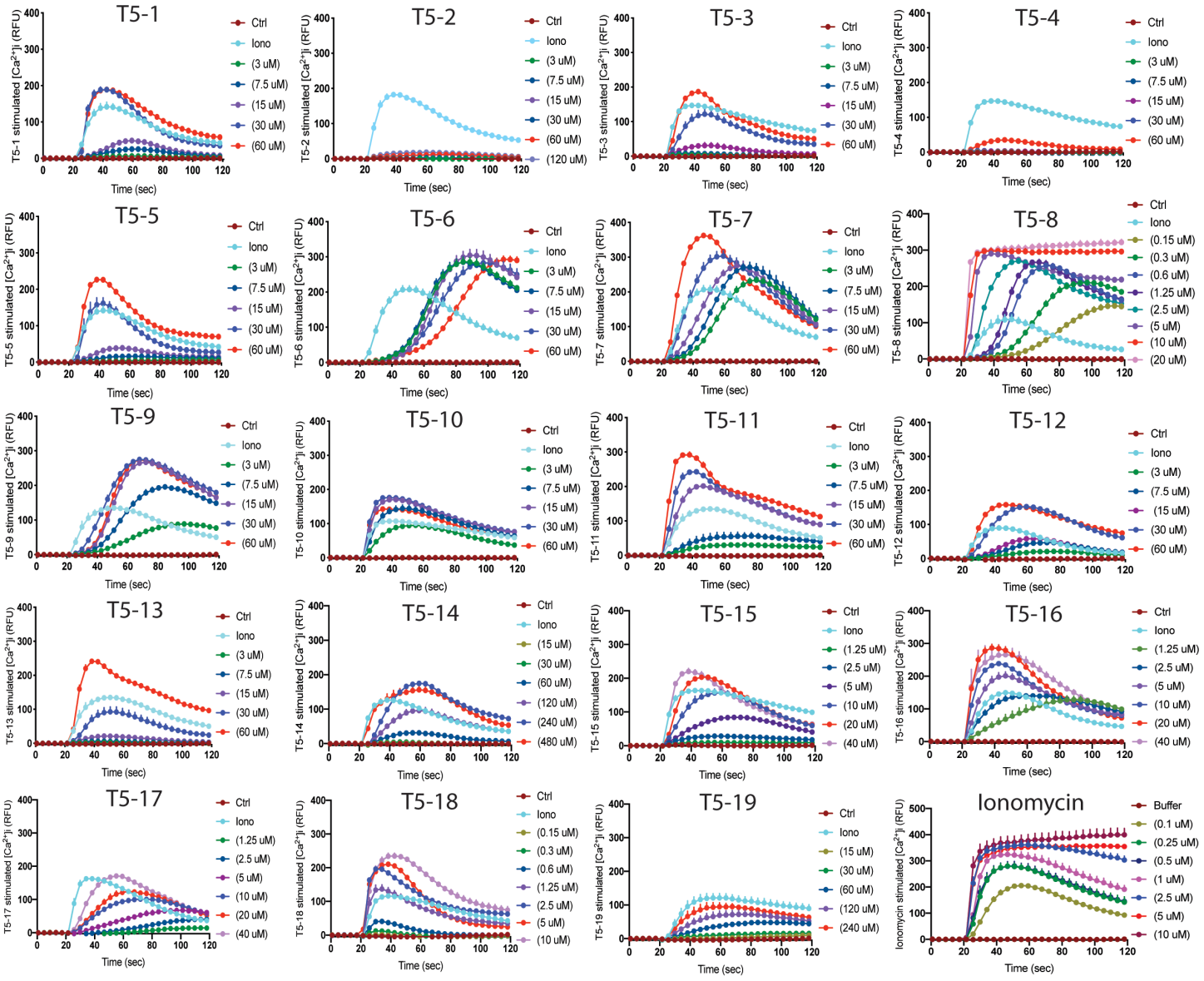
C



procyanidin

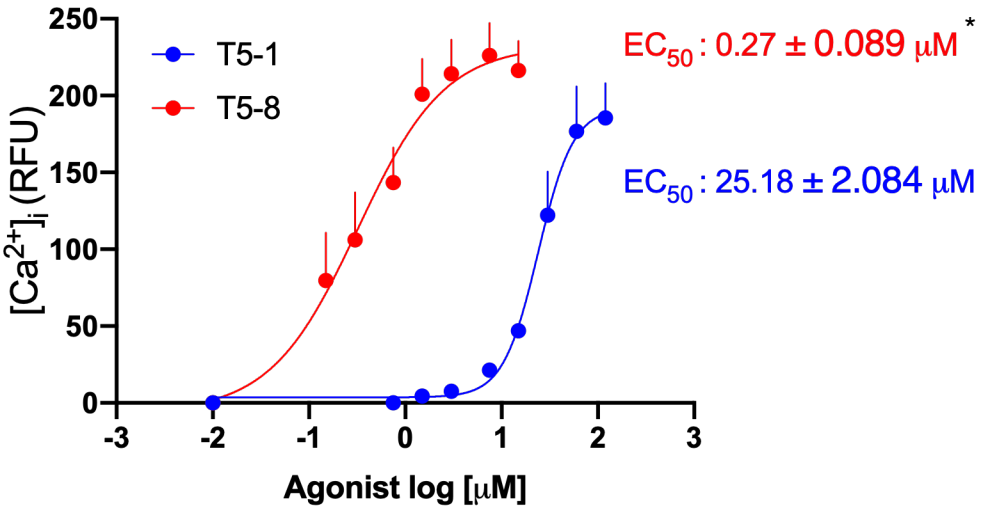
Supporting Information Figure S-1. Structures of the commonly utilized bronchodilator albuterol, a beta-2 adrenergic receptor agonist (A) and two reported TAS2R5 agonists (B, C).

Figure S-2



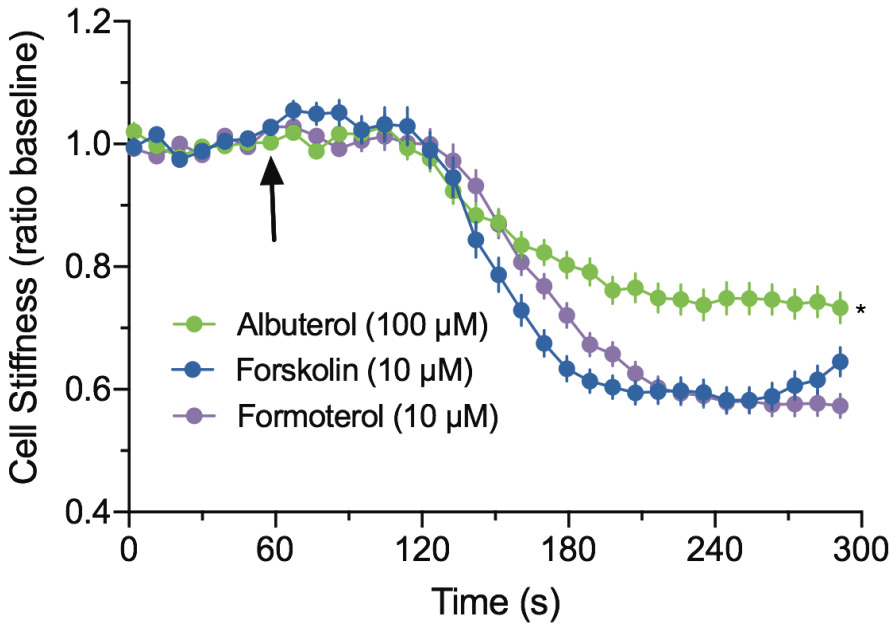
Supporting Information Figure S-2. Representative [Ca2+]i responses in D9-HASM cells over 100 seconds after exposure to the indicated compounds. All compounds except for ionomycin (used as a positive control) are potential TAS2R5 agonists. See Figure 2 showing the mean maximal response obtained from each concentration of agonist.

Figure S-3



Supporting Information Figure S-3. [Ca2+]i responses in primary HASM cells with T5-1 and T5-8 TAS2R5 agonists. Cultured primary HASM cells at passages 5-7 derived from a non-asthmatic lung were exposed to the indicated concentrations of the agonists. Results are from 4 independent experiments. *, EC50 P <0.01 vs T5-1

Figure S-4



Supporting Information Figure S-4. HASM relaxation responses to non-TAS2R5 agonists measured by MTC. Cells were exposed to the beta-agonists albuterol (partial agonist), formoterol (full agonist), and the direct activator of adenylyl cyclase, forskolin. Results are from 200-300 measurements per condition. *, peak maximal response less than formoterol, $P<0.01$