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The Molecular Receptive Ranges of Human TAS2R Bitter Taste Receptors

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Abstract

Humans perceive thousands of compounds as bitter. In sharp contrast, only ~25 taste 2 receptors (TAS2R) bitter taste receptors have been identified, raising the question as to how the vast array of bitter compounds can be detected by such a limited number of sensors. To address this issue, we have challenged 25 human taste 2 receptors (hTAS2Rs) with 104 natural or synthetic bitter chemicals in a heterologous expression system. Thirteen cognate bitter compounds for 5 orphan receptors and 64 new compounds for previously identified receptors were discovered. Whereas some receptors recognized only few agonists, others displayed moderate or extreme tuning broadness. Thus, 3 hTAS2Rs together were able to detect ~50% of the substances used. Conversely, though 63 bitter substances activated only 1–3 receptors, 19 compounds stimulated up to 15 hTAS2Rs. Our data suggest that the detection of the numerous bitter chemicals is related to the molecular receptive ranges of hTAS2Rs.

Key words: calcium imaging, gustation, heterologous expression, taste

Introduction

Bitter taste is innate and triggers stereotypical behavioral outputs leading to rejection (Steiner 1973; Chandrashekar et al. 2006; Beauchamp and Mennella 2009). Although a clear correlation between bitterness and toxicity has not been established (Glendinning 1994), it is generally believed that this taste quality prevents mammals from intoxication by avoiding ingestion of potentially harmful food constituents (Lindemann 1996; Drewnowski and Gomez-Carneros 2000). Many bitter substances are of genuine plant origin, but others derive from animals or are generated during the processing, aging, or spoilage of food (Kingsbury 1964; Drewnowski and Gomez-Carneros 2000; Murata and Sata 2000). A reliable inventory of bitter molecules does not exist, but estimates are in tens of thousands because, for natural bitter compounds alone, some 10% of the plant species may synthesize toxic secondary metabolites and that ~2500 plant species alone contain cyanogenic glycosides, most of which are bitter (Kingsbury 1964; Zagrobelny et al. 2004). Other rich sources of bitter compounds are the Maillard and fermentation reactions (Belitz and Wieser 1985; Hofmann 2005; DuBois et al. 2008), whereas chemical synthesis has provided unique bitter chemotypes (Fox 1932). Bitter compounds are not only numerous but also structurally diverse. They include, but are not limited to, hydroxy fatty acids, fatty acids, peptides, amino acids, amines, amides, azacycloalkanes, *N*-heterocyclic compounds, ureas, thioureas, carbamides, esters, lactones, carbonyl compounds, phenols, crown ethers, terpenoids, secoiridoids, alkaloids, glycosides, flavonoids, steroids, halogenated or acetylated sugars, and metal ions (Belitz and Wieser 1985; DuBois et al. 2008).

Bitter compounds are detected by a specific subset of taste receptor cells localized in the mouth and characterized by the expression of members of the *TASTE 2 Receptor* (*TAS2R or*

T2R) gene family encoding bitter taste receptor candidates (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000; Mueller et al. 2005; Behrens et al. 2007). TAS2Rs comprise a specific family within the superfamily of G protein–coupled receptors (Roper 2007; Behrens and Meyerhof 2009). Depending on the species, vertebrate genomes contain between 3 *TAS2R* genes in chickens and up to 50 in amphibians with ~25 genes in the human genome (Shi and Zhang 2009). This comparatively small number of *TAS2R* genes raises the question as to how vertebrates can perceive as bitter such a large number of chemically diverse bitter substances with such a limited number of receptors.

Previous studies have used functional expression in engineered cell lines to identify the cognate agonists of TAS2Rs. The first member of the family to be functionally characterized was the mouse T2R5 (Chandrashekar et al. 2000). Within 27 bitter tasting chemicals assayed, only cycloheximide activated this receptor. Similar data were obtained also for mT2R8 and hTAS2R4 that responded to propylthiouracil and denatonium benzoate. The data suggested initially that TAS2Rs are narrowly tuned to detect definite bitter substances, though mT2R8 and hTAS2R4 showed a limited degree of promiscuity. However, subsequent functional studies in humans revealed that other bitter taste receptors, like hTAS2R16 and hTAS2R38, are more broadly tuned to recognize numerous compounds possessing either a β-glucopyranoside or a NCS moiety as important common chemical groups responsible for mediating receptor–agonist interactions (Bufe et al. 2002, 2005). Further studies revealed extreme examples for broad agonist spectra of activation for hTAS2R7, hTAS2R14, and hTAS2R46 (Behrens et al. 2004; Brockhoff et al. 2007; Sainz et al. 2007). Agonists for these receptors, and hTAS2R43 and hTAS2R44 as well, lacked any apparent common chemical substructure. Moreover, the receptors responded to up to ~69% of the bitter substances tested, even though the selection of compounds was not well balanced (Behrens et al. 2004; Kuhn et al. 2004; Brockhoff et al. 2007; Sainz et al. 2007). These studies also revealed some degree of redundancy regarding the agonist receptor combinations. For instance, strychnine activated hTAS2R7, hTAS2R10, and hTAS2R46 (Bufe et al. 2002; Brockhoff et al. 2007; Sainz et al. 2007), saccharin, acesulfame K, aloin, and aristolochic acid, the closely related TAS2R43 and TAS2R44 (Kuhn et al. 2004; Pronin et al. 2007), and humulones hTAS2R1, hTAS2R14, and hTAS2R40 (Intelmann et al. 2009). However, the extent of such redundancies could not be evaluated because all recent studies employed only single receptors and limited sets of agonists that were not cross-checked with other human taste 2 receptors (hTAS2Rs). These studies collectively raised the question of whether the molecular receptive ranges of TAS2Rs are the major cause for the ability of vertebrates to perceive an exorbitant number of compounds as bitter. In this study, we therefore have systematically investigated the molecular receptive ranges of bitter taste receptors using 104 natural or synthetic bitter chemicals to challenge all the 25 human TAS2Rs in transfected cells.

Materials and methods

Functional expression studies of hTAS2Rs

Bitter compounds

All tested compounds were described to taste bitter in psychophysical tests or were selected based on common knowledge. Bitter compounds were purchased from Sigma-Aldrich Chemie GmbH and from LGC Promochem or were isolated as described recently (Czepa and Hofmann 2003; Brockhoff et al. 2007). The compounds were either dissolved in buffer C1 (130 mM NaCl, 5 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 2 mM $CaCl_2$, 10 mM glucose [pH 7.4]) or in a mixture of dimethyl sulfoxide (DMSO) and buffer C1 not exceeding a final DMSO concentration of 0.1% (v/v) to avoid toxic effects on the transfected cells.

Calcium imaging analysis

Functional expression studies were carried out as described earlier (Bufe et al. 2002; Behrens et al. 2004; Kuhn et al. 2004; Brockhoff et al. 2007). In short, we transfected human embryonic kidney (HEK)-293T cells with expression plasmids based on pcDNA5/FRT (Invitrogen) or pEAK10 (Edge BioSystems). The plasmids harbor the hTAS2R coding sequences preceded by the first 45 amino acids of rat somatostatin receptor 3 for cell surface localization and followed by the herpes simplex virus (HSV) glycoprotein D epitope for immunocytochemical detection. Empty pcDNA5/FRT vector was used as negative control. HEK293T cells stably expressing the chimeric G protein subunit Gα16gust44 (Ueda et al. 2003) were transiently transfected with 150 ng plasmid DNA per well 24–26 h after seeding the cells into 96-well plates using 300 nL Lipofectamine 2000 per well (Invitrogen GmbH) according to the manufacturer's instructions. The chimeric G protein α subunit couples activated TAS2Rs to phospholipase C activity, inositol trisphosphate, and mobilization of intracellular calcium.

Twenty-four to 26 h after transfection, cells were loaded with the calcium-sensitive dye Fluo-4-AM (2 μM, Molecular Probes) in serum-free culture medium. Probenecid (Sigma-Aldrich GmbH), an inhibitor of organic anion transport, was added at a concentration of 2.5 mM, minimizing the loss of the calcium indicator dye from the cells. Cellular calcium traces were recorded at 510 nm following excitation at 488 nm by a fluorometric imaging plate reader (FLIPR, Molecular Devices) 1 min before and 11 min after bath application of the test compounds. A second application of 100 nM somatostatin 14 (Bachem), activating the endogenous somatostatin receptor type 2, assessed cell vitality. Because many bitter chemicals are pharmacologically active or are

hydrophobic or amphiphilic in nature, they could potentially interfere with cellular calcium responses. All compounds were, therefore, tested at different concentrations for unspecific calcium responses in untransfected HEK293T Gα16gust44 cells. Based on this pilot experiment, we used maximal compound concentrations that were always lower than those concentrations that generated unspecific responses in the absence of transfected receptor DNAs (Supplementary Table 1).

Determination of half maximal effective concentrations and threshold values of receptor activation

For the calculation of the dose–response curves, the fluorescence changes of mock-transfected cells were subtracted from those of the corresponding receptor-expressing cells. To compensate for differences in cell density, signals were normalized to background fluorescence for each well. Signals of 2–3 wells containing the same receptor-expressing cells and which received the same agonist concentrations of 2 or 3 independent experiments were averaged. Dose–response curves were established by plotting signal amplitudes versus log agonist concentration. The half maximal effective concentrations (EC_{50}) were identified by nonlinear regression using the equation $f(x) = 100/[1 + (EC_{50}/x)^{nH}]$, where x is the agonist concentration and nH the Hill coefficient. All calculations and plots were done in SigmaPlot 9.0.

Several tested compounds elicited a cellular response only at 1 or 2 concentrations preventing us from establishing dose response curves. In such cases, the lowest compound concentration that generated a detectable calcium signal was designated as threshold concentration and used as parameter to measure the potency of an agonist at a receptor.

Immunocytochemistry

In order to determine the extent of receptor expression and cell surface localization, we stained the transfected cells with a primary monoclonal mouse anti-HSV glycoprotein D epitope antibody (Novagen) and a secondary Alexa Fluor 488–labeled goat anti-mouse immunoglobulin G (Molecular Probes). To visualize the cell surface, cells were also stained with biotinylated concanavalin A (Sigma-Aldrich) and rhodamine-labeled avidin (Vector Laboratories). Cells were examined using a confocal laser-scanning microscope Leica TCS-SP2 (Leica).

Results

Bitter compounds

In this study, we employed 58 natural bitter and 46 synthetic bitter compounds previously described in the literature as bitter. Within the natural compounds, secondary metabolites from edible, medicinal, and poisonous plants were considered, as exemplified by L-sulforaphane from broccoli (*Brassica oleracea* L.), colchicine from meadow saffron (*Colchicum autumnale* L.), and picrotoxinin from fishberry (*Anamirta cocculus* L.). The selection of natural products encompasses compounds characterized by different levels of human exposure, maximal for those of dietary origin and minimal from those derived from poisonous plants. The presence of all major classes of natural products (alkaloids, vitamins, terpenoids, steroids, acetogenins, phenolic glycosides, flavonoids, and arylheptanoids) testifies how widespread is bitterness in plant secondary metabolites. The intensely bitter microbial antibiotics chloramphenicol and erythromycin have also been included in the selection. The synthetic bitter compounds selected are of various origin but related to human exposure (drugs, health care items, detergents, body care items, and cosmetics), and their structural diversity, from ammonium salts to sulfonamides, phenolics, and heterocyclics, is a testament to the wide distribution of bitterness within the chemical space.

Identification of agonists for orphan receptors hTAS2R3, hTAS2R5, hTAS2R13, hTAS2R39, and hTAS2R49

In order to elucidate the molecular receptive ranges of the 25 human TAS2R bitter taste receptors and to examine the extent of overlap of their agonist spectra, we expressed these receptors individually by transient transfection in HEK293T cells that also stably express the chimeric G protein Gα16gust44 (Ueda et al. 2003). As there are numerous polymorphisms present in the *hTAS2R* genes, Supplementary Table 2 shows the amino acid sequences of the TAS2Rs that we used. Changes in intracellular calcium concentrations were detected by a fluorometric imaging plate reader. We administered all 58 natural and 46 synthetic compounds at various concentrations by bath application to the 25 different receptor-expressing cell populations. This procedure made it possible not only to identify the cognate agonists for the 25 receptors but also to assess which receptors were unresponsive and thus unlikely to mediate a physiological response to a definite bitter stimulus.

The first important result of this experimental approach is the identification of agonists for 5 hTAS2Rs that had previously remained orphan (Figure 1, Tables 1 and 2; Supplementary Tables 1 and 3). Human TAS2R3 and TAS2R5 recognized only single synthetic compounds, the antimalaria drug chloroquine and 1,10-phenanthroline, a heterocyclic organic iron chelator, respectively (Figure 1A,B, Table 2). Chloroquine activated hTAS2R3 with an EC_{50} value of 172 ± 29 μM (Supplementary Table 3). Human TAS2R5 was sensitive to 1,10-phenanthroline in the range from 0.1 to 1 mM. Two potent artificial bitter substances, diphenidol and denatonium benzoate, activated hTAS2R13-expressing cells with threshold concentrations of 30 μM, respectively (Figure 1C, Table 2; Supplementary Table 1). Human TAS2R39 was sensitive to 11 different natural and synthetic substances (Figure 1D, Tables 1 and 2; Supplementary Table 1). Figure 1D shows calcium signals typical of cells expressing this receptor observed after stimulation with

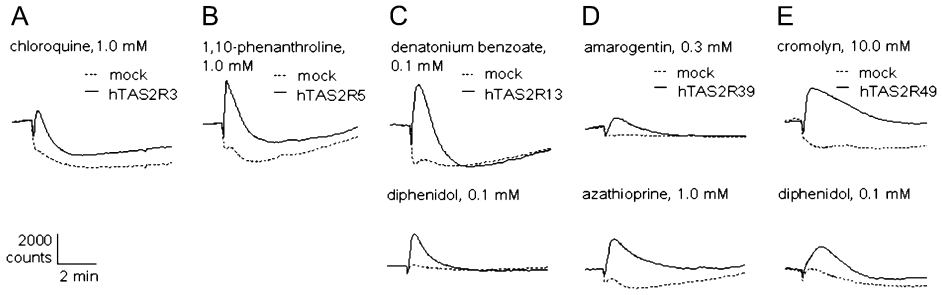

Figure 1 Identification of agonists for hTAS2R3 **(A)**, hTAS2R5 **(B)**, hTAS2R13 **(C)**, hTAS2R39 **(D)**, and hTAS2R49 **(E)**. Receptor-expressing cells were loaded with the calcium indicator and fluorescence emissions recorded before and after exposure of the cells to the indicated bitter substances (solid lines). Responses of mock-transfected cells to the same concentration of the substances are also shown as negative control (dashed lines).

amarogentin or azathioprine. The other activators of hTAS2R39 are thiamine HCl, chloroquine, chlorpheniramine, denatonium benzoate, diphenidol, acetaminophen, quinine HCl, chloramphenicol, or colchicine (Figure 3, Tables 1 and 2; Supplementary Table 1). Finally, we found that cells expressing hTAS2R49 responded to the synthetic chemical cromolyn with an EC_{50} value of 45 ± 25 μM (Figure 1E, Table 2; Supplementary Table 3) and also to diphenidol with a threshold of 0.1 mM (Table 2; Supplementary Table 1). Interestingly, the EC_{50} value of cromolyn for hTAS2R49 is 100-fold lower than that for hTAS2R7 (4.5 ± 1.6 mM; Supplementary Table 3) and below the concentration range required to stimulate hTAS2R43. This indicates that hTAS2R49 is the most sensitive and thus likely the most important taste receptor to detect cromolyn (cromoglycin acid), a synthetic analogue of the natural chromone khellin capable to potently block the secretion of chemical mediators from sensitized mast cells.

Additional agonists for known receptors and receptors that remain orphan

Our functional expression studies also identified numerous new agonists (indicated by + in Tables 1 and 2) for 14 receptors that have previously been assigned some cognate bitter substances (marked by # or § in Tables 1 and 2). Sixty-four substances, 32 natural and 32 synthetic compounds, activated these 14 receptors. Another important observation of the present study is that receptors previously shown to have clear agonist specificities, that is, those that are activated by agonists with common chemical groups, were also sensitive to substances lacking such common groups. The β-glucopyranoside receptor, hTAS2R16, also responded to diphenidol, a diphenyl-piperidinylbutanol which lacks the β-glycosidic configuration otherwise required for activating hTAS2R16 (Bufe et al. 2002). The same compound also activated hTAS2R38, the thiourea/isothiocyanate receptor (Bufe et al. 2005) (Figure 2A). This receptor responded also to the nortriterpene limonin from lemon, orange, or grapefruit, to the roast flavor product ethylpyrazine, and to synthetic substances such as caprolactam, that is, hexanolactam and to the antihistaminic chlorpheniramine (Table 2). All these compounds lack the NCS motif typical of glucosinolates and isothiocyanates, as well as their enzymatic transformation products. Collectively, the data suggest that the promiscuity of TAS2Rs appears to be greater than anticipated (Bufe et al. 2002, 2005).

Notably, we failed to identify agonists for hTAS2R9, hTAS2R41, hTAS2R42, hTAS2R45, hTAS2R48, or hTAS2R60 by any of the 104 bitter substances although our immunocytochemical data show that, with the exception of hTAS2R9, these receptors are visibly expressed and found partly at the cell surface (Supplementary Figure 1). Human TAS2R41, hTAS2R42, hTAS2R45, and hTAS2R60 express well and are found partly at the cell surface, suggesting that our failure to identify their cognate bitter compounds is not due to insufficient expression levels. For hTAS2R48, we see only marginal expression. Thus, in these 2 cases, poor expression may account for our inability to identify corresponding agonists. However, it must be emphasized that 3 agonists, ofloxacin, procainamide, and pirenzapine, have been identified for hTAS2R9 recently (Dotson et al. 2008), compounds that have not been employed in the present study. The use of a nonfunctional hTAS2R9 variant probably prevented us from detecting cognate bitter chemicals. Our variant of hTAS2R9 contains a valine in position 187. This residue disrupts the function of hTAS2R9 normally having an alanine in this position (Dotson et al. 2008). Thus, 5 receptors, hTAS2R41, hTAS2R42, hTAS2R45, hTAS2R48, and hTAS2R60, must still be considered orphan G protein-coupled receptors (GPCRs) (Tables 1 and 2).

Molecular receptive ranges of TAS2Rs

The functional expression studies also revealed obvious differences in the breadth of agonist spectra of the various hTAS2Rs, the "extrema" being hTAS2R3 and hTAS2R5, which responded only to single compounds, and hTAS2R14, which, conversely, was activated by 33 out of the 104 compounds assayed (Tables 1 and 2). A rough classification according to the receptor's breadth of tuning would sort the receptors into 4 groups. The first group consists of

Table 1 Response profiles of 25 hTAS2Rs stimulated with 58 natural bitter compounds

hTAS2R compound	1	3	4	5	7	8	9	10	13	14	16	38	39	40	41*	42*	43	44	45*	46	47	48*	49	50	60*	n.r.	mock
Absinthin	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	#	#	–	–	–	–	4	–
2-Acetylpyrazine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Aloin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	#	§	–	–	–	–	–	–	–	2	–
Allylisothiocyanyte	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Amarogentin	+	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	#	–	–	#	#	–	–	#	–	7	–
Amygdalin, D	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Andrographolide	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	#	#	–	–	#	–	3	–
Arborescin	+	–	+	–	–	–	–	+	–	+	–	–	–	–	–	–	+	–	–	#	–	–	–	–	–	6	–
Arbutin	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Arglabin	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	+	–	–	#	–	–	–	–	–	4	–
Aristolochic acid	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	–	#	#	–	–	–	–	–	–	–	3	–
Artemorin	–	–	+	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	#	+	–	–	–	–	5	–
Brucine	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	2	–
Campher	–	–	+	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	4	–
Caffeine	–	–	–	–	+	–	–	+	–	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	5	–
Cascarillin	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	#	+	–	–	–	–	5	–
Chloramphenicol	+	–	–	–	–	+	–	+	–	–	–	–	+	–	–	–	+	–	–	#	–	–	–	–	–	6	–
Cnicin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	1	–
Colchicine	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	3	–
Coumarin	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Crispolide	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	1	–
Cucurbitacin B	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Cucurbitacin E	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Curcumin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Digitonin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Emetine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Erythromycin	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Ethylpyrazine	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Falcarindiol	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	2	–
Ginkgolide A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Grossheimin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	#	–	–	–	–	–	2	–
Helicin	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	+	–	–	–	–	–	–	–	–	2	–
Humulone isomeres	#	–	–	–	–	–	–	–	–	#	–	–	–	#	–	–	–	–	–	–	–	–	–	–	–	3	–
Limonin	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Naringin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Neohesperidine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Nicotin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Nimbin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Noscapine	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–

Table 1 Continued

hTAS2R compound	1	3	4	5	7	8	9	10	13	14	16	38	39	40	41*	42*	43	44	45*	46	47	48*	49	50	60*	n.r.	mock
Ouabain	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Papaverine	–	–	–	–	#	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–
Parthenolide	+	–	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	+	–	#	–	–	–	–	–	7	–
Picrotoxinin	+	–	–	–	–	–	–	+	–	#	–	–	–	–	–	–	–	–	–	#	+	–	–	–	–	5	–
Phenylethyl isothiocyanate	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Quassin	–	–	+	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	5	–
Quinine	–	–	+	–	+	–	–	+	–	+	–	–	+	+	–	–	+	+	–	+	–	–	–	–	–	9	–
Riboflavin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
D-salicin	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Sinigrin	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Solanine, alpha	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Solanidine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Strychnine	–	–	–	–	§	–	–	#	–	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	3	–
L-sulforaphane	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Taurine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Tatridin B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	1	–
Thiamine	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Thujon, (–)-a-	–	–	–	–	–	–	–	+	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Yohimbine	+	–	+	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	5	–

*, Receptors remaining orphan; n.r., number of receptors identified; mock, results obtained with mock-transfected cells at the highest concentration employed; +, response; –, no response; #, previously known agonist confirmed in the present study; §, previously known agonist not confirmed in the present study (Chandrashekar et al. 2000; Bufe et al. 2002, 2005; Kim et al. 2003; Behrens et al. 2004, 2009; Kuhn et al. 2004; Pronin et al. 2004, 2007; Brockhoff et al. 2007; Sainz et al. 2007; Maehashi et al. 2008; Intelmann et al. 2009).

hTAS2R16 and hTAS2R38 (Figure 2, Tables 1 and 2; Supplementary Tables 1 and 3). Although only a limited number of β-glucopyranosides and NCS-containing chemicals have been assayed (Bufe et al. 2002, 2005; Soranzo et al. 2005; Hinrichs et al. 2006), the data suggest that, given the significant percentage of plant species that produce these substances, the actual number of dietary agonists for these receptors is very high (Kingsbury 1964; Bones and Rossiter 1996). Despite this, we found that both receptors also responded to compounds lacking these common motifs, although the promiscuity of TAS2R16 and hTAS2R38 appears restricted (Figure 2, Tables 1 and 2; Supplementary Tables 1 and 3). A good example illustrating the specificity of the 2 TAS2Rs is sinigrin, a glucosinolate contained in cruciferous plants like cabbage, brussels sprouts, and cauliflower to which both receptors respond. Human TAS2R38 apparently does so because it recognizes the presence of the NCS motif in the aglycon, whereas hTAS2R16 recognizes this compound as a β-glucopyranoside (Table 1).

The second group encompasses hTAS2R3, hTAS2R5, hTAS2R8, hTAS2R13, hTAS2R49, and hTAS2R50. Only 9 compounds activated these receptors, with 1–3 agonists per receptor (Tables 1 and 2; Supplementary Tables 1 and 3). In general, the limited agonist spectrum of these receptors seems to be correlated to possible common structural properties of their agonists that might be important for receptor–agonist interactions. Thus, the TAS2R8 agonists chloramphenicol and denatonium benzoate share a nitrogen function, a carbonyl group (in the benzoate counterion of the ammonium salt), and an aromatic ring. These features are also evident in saccharin, another hTAS2R8 agonist. The TAS2R50 agonists, amarogentin and andrographolide, share a lactone ring and an aliphatic ring bearing hydroxyl groups, whereas the TAS2R49 agonists diphenidol and cromolyn have aromatic rings and a hydroxyl group bound to an aliphatic structure. Conversely, the TAS2R13 agonists diphenidol and denatonium benzoate only share the presence of a nitrogen atom in vicinity to a hydroxyl group.

The third group comprises hTAS2R1, hTAS2R4, hTAS2R7, hTAS2R39, hTAS2R40, hTAS2R43, hTAS2R44, and hTAS2R47. These receptors responded to 6–16 compounds (Figure 2, Tables 1 and 2; Supplementary Tables 1 and 3). Altogether, 40 compounds could activate these receptors, 24 natural and 16 synthetic, suggesting that this class of

Table 2 Response profiles of 25 hTAS2Rs stimulated with 46 synthetic bitter compounds

hTAS2R compound	1	3	4	5	7	8	9	10	13	14	16	38	39	40	41*	42*	43	44	45*	46	47	48*	49	50	60*	n.r.	mock
Acesulfame K	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	#	#	–	–	–	–	–	–	–	2	–
Acetaminophen	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Acetylthiourea	–	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Antipyrin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Azathioprine	–	–	+	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	5	–
Benzamide	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Benzoin	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Caprolactam	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Carisoprodol	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	2	–
Chlorhexidine	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Chloroquine	–	+	–	–	§	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	4	–
Chlorpheniramine	–	–	+	–	+	–	–	+	–	+	–	+	+	+	–	–	–	–	–	+	–	–	–	–	–	8	–
Cromolyn	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	3	–
Cycloheximide	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Dapsone	–	–	+	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	3	–
Denatonium benzoate	–	–	#	–	–	+	–	+	+	–	–	–	+	–	–	–	#	–	–	#	#	–	–	–	–	8	–
Dextromethorphan	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Dihydrophenanthrene	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Dimethyl thioformamide	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Diphenhydramine	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	2	–
Diphenidol	+	–	+	–	+	–	–	+	+	+	+	+	+	+	–	–	+	+	–	+	+	–	+	–	–	15	–
Diphenylthiourea	+	–	–	–	–	–	–	–	–	+	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–
Divinylsulfoxid	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Doxepin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
N-ethylthiourea	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
N,*N*′-ethylene thiourea	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Famotidine	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	2	–
Fenspiride	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Flufenamic acid	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Haloperidol	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Hydrocortisone	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	1	–
4-Hydroxyanisol	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Methimazole	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
4(6)-Methyl-2-thiouracil	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Methylthiourea	–	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Metronidazole	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Naphazoline	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–
Orphenadrine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	1	–
Oxolinic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	–

Table 2 Continued

hTAS2R compound	1	3	4	5	7	8	9	10	13	14	16	38	39	40	41*	42*	43	44	45*	46	47	48*	49	50	60*	n.r.	mock
1,10-Phenanthroline	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Phenylthiocarbamide	–	–	–	–	–	–	–	–	–	–	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Propylthiouracil	–	–	§	–	–	–	–	–	–	–	–	#	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Saccharin	–	–	–	–	–	§	–	–	–	–	–	–	–	–	–	–	#	#	–	–	–	–	–	–	–	3	–
Sodium benzoate	–	–	–	–	–	–	–	–	–	#	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Sodium cyclamate	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–
Sodium thiocyanate	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–

*, Receptors remaining orphan; n.r., number of receptors identified; mock, results obtained with mock-transfected cells at the highest concentration employed; +, response; –, no response; #, previously known agonist confirmed in the present study; §, previously known agonist not confirmed in the present study (Chandrashekar et al. 2000; Bufe et al. 2002, 2005; Kim et al. 2003; Behrens et al. 2004, 2009; Kuhn et al. 2004; Pronin et al. 2004, 2007; Brockhoff et al. 2007; Sainz et al. 2007; Maehashi et al. 2008).

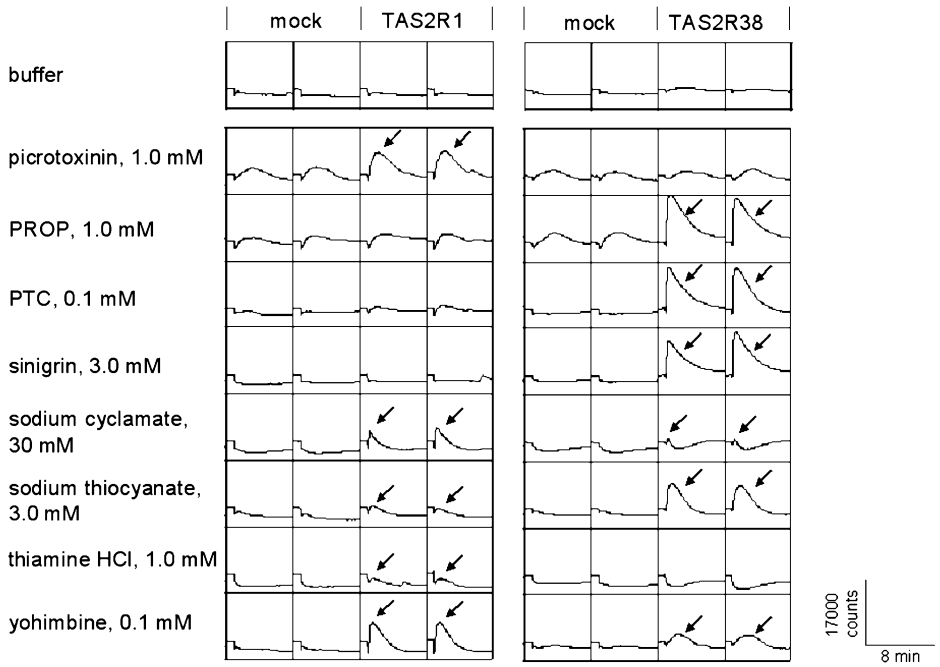

Figure 2 hTAS2R respond to several bitter compounds. Cells were loaded with the calcium indicator and fluorescence emissions recorded before and after exposure of the cells to the indicated bitter substances. The panels show FLIPR recordings of calcium responses of HEK293T Gα16gust44 cells expressing hTAS2R38 or hTAS2R1. M, mock-transfected cells. Arrows point to calcium responses.

receptors has a penchant for natural compounds. Overall, these receptors have quite broad agonist spectra, with the lack of clear common motifs that could be responsible for a specific recognition.

Finally, hTAS2R10, hTAS2R14, and hTAS2R46, which responded to 32, 33, and 28 compounds, respectively, belong to the fourth group (Tables 1 and 2; Supplementary Tables 1 and 3). Common structural motifs in these chemicals are not obvious. These receptors are very promiscuous, showing extremely wide molecular receptive ranges. There appears also to be a slight preference of these receptors for natural compounds because 32 of them activated these receptors, which were in turn sensitive to only 22 synthetic compounds. These data intriguingly suggest that ~50% of the bitter compounds considered can be detected with only these 3 receptors. Conversely, and with only 2 exceptions, that is, hTAS2R3 and hTAS2R5, human TAS2Rs are activated by several compounds, and the majority of hTAS2Rs respond to numerous compounds (Figure 2, Tables 1 and 2).

Matching compounds and receptors

The data presented in Tables 1 and 2 allow several important observations to be made on the number of agonists that activate the individual receptors. Thus, many bitter

compounds activate several TAS2Rs (Figure 3), the supreme example being diphenidol, a compound capable of stimulating 15 different receptors (Table 2; Supplementary Table 1). Other promiscuous ligands are quinine (9 receptors), chlorpheniramine and denatonium benzoate (8 receptors), amarogentin (7 receptors), arborescin and chloramphenicol (6 receptors), azathioprine, caffeine, and cascarillin (5 receptors), arglabin and absinthin (4 receptors), cromolyn, colchicines, and papaverin (3 receptors), as well as diphenhydramine and famotidine (2 receptors). Despite these observations, Figure 4 as well as Tables 1 and 2 clearly show that the majority of compounds activate only 1 (32 compounds), 2 (21 compounds), or 3 receptors (10 compounds), with only 19 substances activating 4 or more receptors. Almost half of the 82 chemicals that found a receptor were specific for only 1 TAS2R. Tables 1 and 2 also reveal a difference between natural and synthetic compounds. Whereas 14 natural compounds activated 4 or more hTAS2Rs, only 5 synthetic compounds did so.

It is also interesting to consider the concentration ranges of agonists necessary to activate the receptors (Figure 3B; Supplementary Tables 1 and 3). First, we must emphasize that, in many instances, EC_{50} values could not be determined. In almost all cases, this was prevented by the property of many bitter compounds to generate artificial calcium responses in the absence of transfected hTAS2Rs at high concentrations. Probably, activation of other cellular effectors or interference with the integrity of the plasma membrane account for this limitation. It is unlikely that endogenous TAS2Rs mediate these artificial responses because in most cases, they show a different kinetic than responses mediated by G protein–coupled receptors. We also did not detect TAS2R1, TAS2R16, or TAS2R38 mRNA in HEK293 cells by reverse transcription–polymerase chain reaction. A low-level expression of some TAS2Rs cannot, however, completely be excluded. Despite these restrictions, we could determine several EC_{50} values (Supplementary Table 3) or, in other cases, obtained threshold concentrations, defined here as the lowest concentration that elicited a calcium response (Supplementary Table 1). Quinine, for instance, activates all its 9 hTAS2Rs in the same concentration range, displaying threshold values of activation of 10 μM for all of them

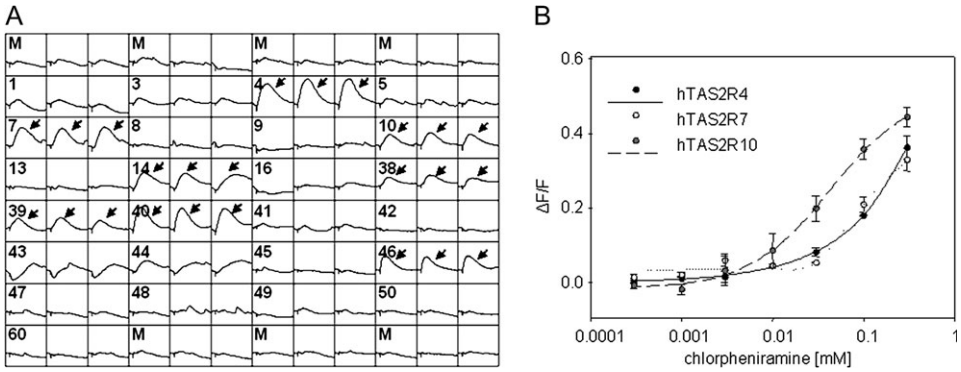

Figure 3 One compound can activate several receptors. Cells were loaded with the calcium indicator and fluorescence emissions recorded before and after exposure of the cells to 0.1 mM chlorpheniramine. The panels show FLIPR recordings of calcium traces of HEK293T Gα16gust44 cells expressing hTAS2Rs **(A)** and some selected dose–response curves **(B)**. At this concentration, chlorpheniramine activates 8 hTAS2Rs. M, mock-transfected cells. Arrows point to calcium responses.

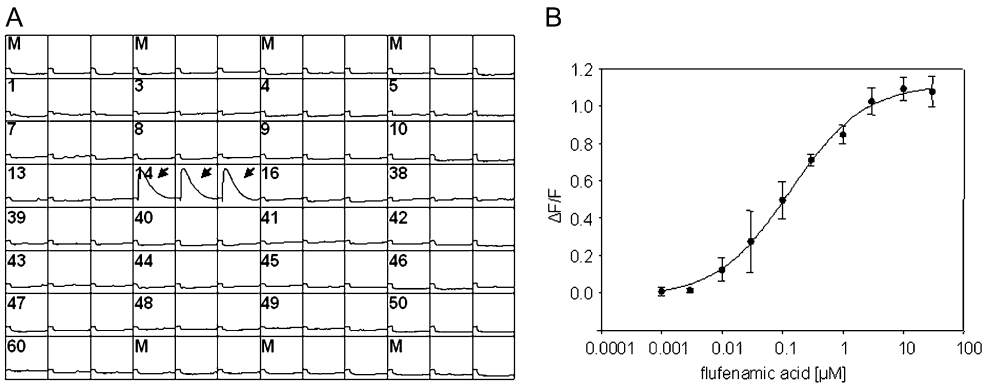

Figure 4 Some compounds activate only 1 receptor. Cells were loaded with the calcium indicator and fluorescence emissions recorded before and after exposure of the cells to 0.03 mM flufenamic acid. **(A)**, The panels show FLIPR recordings of calcium traces of HEK293T Gα16gust44 cells expressing the indicated hTAS2Rs or of mock-transfected cells (M). The dose–response curve for flufenamic acid in cells expressing hTAS2R14, the single responsive receptor, is shown in **(B)**. Arrows point to calcium responses. Flufenamic acid (0.03 mM) activates only 1 receptor, hTAS2R14.

(Supplementary Table 1). In marked contrast, denatonium benzoate showed a concentration range of activation that spanned ~5 orders of magnitude (Supplementary Table 1), with threshold concentrations of 100 nM, 3, 30, 100, 300 μM, and 1 mM for the activation of hTAS2R47, TAS2R10, hTAS2R13 or hTAS2R46, hTAS2R39, hTAS2R4 or hTAS2R43, and hTAS2R8, respectively. Concentration ranges that span 2 orders of magnitude are often seen for hTAS2Rs and their agonists (Supplementary Tables 1 and 3). Despite our efforts, we did not find a receptor for 15 out of the 58 naturally occurring and 7 out of the 46 synthetic bitter substances assayed (Tables 1 and 2; Supplementary Table 4).

Discussion

We have investigated the activation pattern of bitter taste receptors by challenging the 25 hTAS2Rs with a collection of 104 bitter chemicals. This strategy made it possible to sort responders from nonresponders, whereas previous studies only examined single receptors and comparatively small agonist collections. The present and the previous studies identified cognate bitter compounds for 20 of the ~25 hTAS2Rs (Chandrashekar et al. 2000; Bufe et al. 2002, 2005; Kim et al. 2003; Behrens et al. 2004; Kuhn et al. 2004; Pronin et al. 2004, 2007; Brockhoff et al. 2007; Sainz et al. 2007; Dotson et al. 2008; Maehashi et al. 2008; Intelmann et al. 2009), providing in-depth insight into the functional properties of hTAS2Rs.

The 20 hTAS2Rs deorphaned to date appear to be sufficient to extrapolate the general behavior of the hTAS2R family. Thus, 19 (we did not activate hTAS2R9 as mentioned before) of the 20 receptors allow the detection of ~80% of the bitter chemical library. Supplementary Figure 2 presents a selection of cognate bitter substances for the 19 TAS2Rs to illustrate their chemical diversity. The identified TAS2Rs include sensors for many compounds commonly present in our food, such as glycosides, alkaloids, terpenoids, and flavonoids. Overall, natural and synthetic compounds appear to be sensed equally well; we found receptors for 43 natural and 39 synthetic compounds. However, some receptors, including hTAS2R5, hTAS2R8, hTAS2R13, and hTAS2R49, apparently show a bias for synthetic compounds, whereas others, such as hTAS2R1, hTAS2R4, hTAS2R7, hTAS2R39, hTAS2R40, hTAS2R43, hTAS2R44, and hTAS2R47, seem to prefer natural agonists.

A couple of further points surfaced during these studies. First, hTAS2Rs are heterogenous in terms of tuning broadness. Whereas some receptors recognize only a single or few compounds, others respond to numerous chemicals. Intriguingly, 3 receptors (hTAS2R10, hTAS2R14, and hTAS2R46) showed extremely wide agonist promiscuity, with combined recognition of ~50% of the bitter compounds used and the capability of each receptor to detect ~30% of the bitter chemicals of the challenging library. Most interestingly, from an evolutionary perspective, all 3 receptors seem to share exceptional roles during mammalian development. Both hTAS2R10 and hTAS2R14 are the only human *TAS2R* genes placed at the roots of mouse-specific *tas2r* gene expansions in an interspecies neighbor joining tree (cf., Figure 2; Shi et al. 2003). Whereas *hTAS2R14* is the only human *TAS2R* gene related to mouse *tas2r* cluster 1, *hTAS2R10* is the single human gene related to mouse tas2r cluster 2. Hence, one could speculate that the exceptionally broad tuning of these 2 receptors may reflect the combined agonist specificities of an entire cluster of mouse genes. Exactly, the opposite is true for the *hTAS2R46* gene. In this case, *hTAS2R46* is in the center of the single human-specific *TAS2R* gene expansion, and one could speculate that it might resemble the multifunctional ancestral gene at the base of more specialized descendant hTAS2Rs. If this is true, one could hypothesize that some phylogenetically old *TAS2R* genes have acquired a broadening of their agonist specificities during evolution, perhaps, until the necessary accuracy of recognition required species-specific gene duplication events for well-balanced selectivity. After such gene expansion events, the functionally redundant ancestral gene may become obsolete and be removed from the group of functional genes by mutation. This idea is supported by the high number of pseudogenes related to the human-specific *TAS2R* gene cluster. In addition to *hTAS2R46*, which is a segregating pseudogene (Kim et al. 2003), 4 pseudogenes are associated with this hTAS2R gene subfamily representing approximately half of the entire human *TAS2R* pseudogene repertoire. Like hTAS2R16 and hTAS2R38 agonists, cognate compounds for hTAS2Rs may have structural motifs that likely determine receptor–agonist interaction (Bufe et al. 2002, 2005; Brockhoff et al. 2007) or may lack such motifs (Behrens et al. 2004; Brockhoff et al. 2007). Alternatively, common motifs could exist, but these are, at present, not obvious from the heterogenous structural nature of the activators. Anyhow, capitalizing on a structurally diverse library of over 100 bitter compounds, both artificial and natural, it was confirmed that some hTAS2Rs have indeed a selective, albeit not unique, agonist activation pattern, whereas others are more promiscuous. The molecular basis for this promiscuity is currently unknown but will be interesting to investigate because GPCRs generally have a narrow activation tuning (Gilman 1987). Residues in the transmembrane regions and extracellular loops may form the ligand-binding pockets of hTAS2Rs (Pronin et al. 2004), but further information is lacking.

Next, it is also obvious that bitter compounds differ in their capacity to stimulate TAS2Rs. About 50% of the compounds investigated stimulated only 1 hTAS2R, whereas the other half stimulated 2–9 or even 15 receptors. In general, bitter compounds activate various hTAS2Rs in different concentration ranges. Usually, differences are in the range of 10- to 100-fold, but, as in the extreme case of denatonium benzoate, they can also span 5–6 orders of magnitude. The ability to stimulate several receptors could be mediated by

the number and combination of "functional groups" present in the bitter chemicals and/or by the ability of a ligand to form a, still elusive, "general bitter motif" that fits with low affinity in the binding pocket of several hTAS2Rs. The former assumption is supported by the observation that, for instance, amarogentin, denatonium benzoate, yohimbine, chloramphenicol, and parthenolide, activating 7, 8, 5, 6, or 7 hTAS2Rs, respectively, contain not only a variety of polar groups that could function as hydrogen donors/acceptors or engage in polar interactions but also lipophilic elements that can mediate hydrophobic interactions. The latter assumption is supported by observations that, in many instances, hTAS2R activation shows limited concentration dependence (Supplementary Tables 1 and 3) and results in low signal amplitudes, strongly suggesting that many compounds function only as partial agonists (Figures 2 and 3).

The observations that 1 TAS2R can recognize several compounds and, conversely that 1 compound can, in different concentration ranges activate several hTAS2Rs generate a combinatorial activation pattern of hTAS2Rs that is reminiscent of olfactory receptors (Malnic et al. 1999). In the olfactory system, the combinatorial receptor code together with the expression of only 1 type olfactory receptor per sensory neuron underlies the discrimination of odorants (Malnic et al. 1999; Buck 2004). Like olfactory sensory neurons, also bitter taste receptor cells of the gustatory system appear to be functionally distinct as suggested by the different sets of hTAS2Rs they express (Behrens et al. 2007), by their distinct responses to bitter stimulation (Caicedo et al. 2002), by nerve fiber recordings (Dahl et al. 1997), and by recordings from neurons of the "nucleus tractus solitarius" (Geran and Travers 2006). This raises the possibility of discrimination between bitter chemicals. On the other hand, sensory analyses are controversial and have raised doubt on this issue (Yokomukai et al. 1993; Delwiche et al. 2001; Spector and Kopka 2002; Keast et al. 2003; Brasser et al. 2005). What other consequences could a combinatorial activation pattern of hTAS2Rs have? First, we speculate that multiple receptors for the same compound may provide a backup in case mutations disrupt the function of 1 or more hTAS2Rs. This, to the best of our knowledge, would be unprecedented in other, usually more conserved, gene families and may be related to the pressure for rapid evolution of bitter taste receptor genes, which has been shown to be driven by positive selection for at least some hTAS2Rs (Shi et al. 2003; Go et al. 2005; Soranzo et al. 2005; Shi and Zhang 2006; Zhou et al. 2009). Next, simultaneous activation of several receptors by 1 compound could generate a larger cellular or nerve response compared with activation of a single receptor and result in increased bitterness. Finally, different concentration ranges of multiple responding hTAS2Rs to a given substance would probably enable detection of that chemical over a wider concentration range with increased sensitivity compared with a situation in which only 1 receptor responds.

If we focus on the coevolution of bitter taste receptor genes and natural bitter compounds, as artificial bitter substances do not provide evolutionary relevant constraints, the individual detection thresholds for bitter substances could have evolved for different reasons. First, the detection threshold concentrations might be adjusted to match the concentrations at which the substance occurs in nature. The bitter taste of, for example, a plant would then merely serve as an unpleasant taste hallmark for herbivores and perhaps induce avoiding behavior, saving the plant from extensive grazing. Second, the detection threshold may reflect the pharmacological activity/toxicity of the bitter compound and prevent the animal from ingesting harmful doses of such substances. Good examples to differentiate between these 2 possible roles for bitter taste receptors are the seeds of the 2 plants *Strychnos nux vomica* and *Anamirta paniculata*. Both contain high amounts of structurally very similar bitter compounds, 1 highly toxic, 1 less toxic, or nontoxic. *Strychnos* seeds contain about 3.4% of the poisonous bitter alkaloid strychnine as well as ~2.5% of the much less toxic brucine (Rathi et al. 2008), which is identical to strychnine except for 2 additional methoxy groups decorating the benzene ring of that molecule. Whereas the lethal dosage (LD) of strychnine can be as low as 5–10 mg, that of brucine is up to 100–200 times higher (LD = 1000 mg; cf., Hazardous Substances Data Bank (HSDB); http://toxnet.nlm.nih.gov). If the bitter taste of *Strychnos* seeds would only be a recognition tag, both substances should work equally well. However, the most sensitive receptor for the 2 substances, hTAS2R46, recognizes strychnine with ~100 times greater sensitivity (threshold = 0.1 μM) than brucine (threshold = 10 μM), thus matching almost perfectly the difference in toxicity among both substances. Moreover, the sensitivity of hTAS2R46 for strychnine (~3 μM for signal saturation) exceeds by far the sensitivity that would be necessary for simple detection of strychnine in *Strychnos* seeds (3% roughly equals a concentration of 100 mM), indicating that hTAS2R46 is tuned to detect strychnine as sensitively as possible and not just as sensitive as necessary. A strikingly similar example is the seeds of *A. paniculata* (Cocculus indicus), which contain picrotoxin, an equal mixture of the highly toxic picrotoxinin (symptoms of severe poisoning occur at ~20 mg of picrotoxin; cf., HSDB; http://toxnet.nlm.nih.gov) and the nontoxic picrotin. Again, both substances differ only by the presence of a hydroxyl group attached to an isoprenyl group in picrotin, but toxicity differs even more than between strychnine and brucine. The most sensitive human bitter taste receptor we found for both substances is hTAS2R14 recognizing the toxic picrotoxinin (threshold ~3 μM) approximately 10 times more sensitively than the harmless picrotin (threshold ~30 μM). As the seeds, also called "fishberries," contain 1.5–5% of the mixed compound picrotoxin (see Munch and Ponce 1934 and references therein) (equals ~26–86 mM picrotoxinin), maximal activation of hTAS2R14 (~100 μM) is achieved already by traces of the compound.

Our observations also raise a number of intriguing questions. The most obvious are, why some hTAS2Rs resisted deorphanization and, conversely, why cognate hTAS2Rs for all members of the bitter chemical library could not be found. Regarding the first question, it is worth mentioning that hTAS2R genes contain numerous single nucleotide polymorphisms (SNPs) (Kim et al. 2005), some of which generate nonfunctional receptor variants (Kim et al. 2003; Bufe et al. 2005; Pronin et al. 2007; Dotson et al. 2008), whereas SNP-induced changes in agonist spectra of TAS2Rs have not been reported so far. The use of such nonfunctional variants in our deorphanization attempts might have prevented us from detecting cognate agonists. Indeed, as mentioned above, our variant of hTAS2R9 contains the deleterious valine in position 187 (Dotson et al. 2008) and most probably accounts for our failure to find cognate bitter compounds for this receptor. Other possibilities that could account for our inability to deorphanize all hTAS2Rs could be an insufficient expression level. Another possibility would be a lack of G protein coupling in our system for some receptors because different hTAS2Rs might couple differently to G proteins (Wong et al. 1996; Ozeck et al. 2004; Bufe et al. 2005; Sainz et al. 2007) or the limitation of the experimental set up, as testified by differences across studies. For instance, in our studies, saccharin could not activate hTAS2R8, in contrast to data from Pronin et al. (2007). We were also unable to activate hTAS2R7 with brucine, salicin, strychnine, or propylthiocarbamide, whereas Sainz et al. (2007) identified these compounds as hTAS2R7 activators. Most likely, differences between single-cell calcium imaging, FLIPR recordings, guanosine 5′-O-[gamma-thio]triphosphate binding, or cell lines can affect to some extent the outcome of these types of experiments. With regard to the second problem, we argue that the bitter chemicals in question would be unidentified agonists for the orphan receptors. Once the experimental difficulties discussed above are overcome, they could be successfully assigned to one of the orphan receptors. Alternative explanations would be that some compounds could circumvent receptor binding by directly activating G proteins or cellular effectors in taste receptor cells (Naim et al. 1994; Lindemann 2001; Zubare-Samuelov et al. 2005) or use a different receptor system (Zubare-Samuelov et al. 2003; Oliveira-Maia et al. 2009). Still another alternative would be the possible formation of TAS2R oligomerization and the existence of oligomer-specific bitter compounds.

Taken together, our data conclusively suggest that functional diversity of bitter taste receptor cells, and therefore our ability to perceive the enormous number of bitter substances with a limited number of sensors, is linked to the molecular receptive ranges of hTAS2R bitter taste receptors.

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Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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