Functional characterization of human olfactory receptors responding to pyrazine odorants

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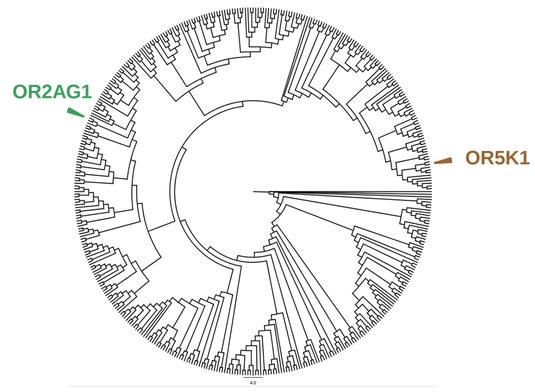


Figure 1. - Absence of paralogy between OR2AG1 and OR5K1 illustrated by their relative distance on the human ORs cladogram.

Table 1.- *In vitro* activity and sensory relationships of OR5K1 and OR2AG1. Description of the organoleptic properties are given by The Good Scents Company. Potency of agonists is measured by the $logEC_{50}$ calculated from concentration-response analyses of an experiment representative of at least two independent experiments. Efficacy of agonists is measured by the E_{max}, or to the highest measured value when an E_{max} plateau is not obtained; values are % of the response induced by 10 µM Forskolin [% FSK] ; '-' = not active.

	Sample name	Structure	OR5K1 - $logEC_{50}$	OR5K1 E_{max} [% FSK]	OR2AG1 - $logEC_{50}$	OR2AG1 E_{max} [% FSK]	Odor description
1	2-Isopropyl-3-methoxypyrazine		6	83	6.7	92	green pea earthy beany
2	2-Methoxy-3-(1-methylpropyl)pyrazine		4.8	42	6.4	83	green musty pea galbanum bell pepper
3	2-Isobutyl-3-methoxypyrazine		5.5	66	6	79	green pea bell pepper galbanum
4	2-Isobutyl-3-methylpyrazine		3.4	40	3	25	caramellic herbal green sugar syrup
5	2-Ethyl-3-methoxypyrazine		5	114	3.8	22	earthy raw potato bell pepper nutty
6	2-Ethylpyridine		4.2	62	3.8	20	green grassy
7	2-Isobutylthiazole		4.5	32	4.2	14	green vegetable musty with a waxy nuance
8	2,3-Diethylpyrazine		4.2	113	3	8	nutty raw green pepper
9	2-Ethyl-3,5(or 6)-dimethylpyrazine		5.5	117	-	-	nutty burnt almonds roasted nuts coffee
10	2,3,5-Trimethylpyrazine		4.7	110	-	-	nutty musty earthy powdery cocoa roasted peanut
11	2-Acetyl-3,5(6)-dimethylpyrazine		4.3	105	-	-	nutty roasted hazelnut caramel popcorn
12	2-Methoxy-3-methyl pyrazine		5.1	99	-	-	nutty roasted almond hazelnut peanut
13	2-methoxy-3,5-dimethylpyrazine		7.4	97	-	-	earthy
14	2,3-Diethyl-5-methylpyrazine		6.8	97	-	-	musty nutty meaty vegetable roasted hazelnut
15	2,4-Lutidine		4.6	84	-	-	smoky phenolic
16	2,6-Dimethylpyrazine		3.6	82	-	-	chocolate cocoa roasted nutty roast beef coffee
17	2-Ethyl-3-methylpyrazine		3.4	78	-	-	nutty peanut musty corn raw earthy bread
18	6-Methylquinoline		4.7	75	-	-	animal leather tonka castoreum tobacco civet fecal
19	2,5-Lutidine		4.5	73	-	-	earthy roasted green
20	2,5-Dimethylpyrazine		3.9	70	-	-	chocolate cocoa roasted nutty roast beef woody grass medical
21	3-Ethylpyridine		4.5	57	-	-	tobacco oakmoss leather
22	Methylisoeugenol		4.7	53	-	-	spicy clove blossom carnation woody

Substitution by $-CH_3$ in R3 or R4 increases activity of **OR5K1**

Substitution in R1 is mandatory for **OR5K1**

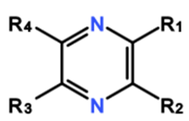
HBA*: ↑ **OR5K1** ↑ **OR2AG1**

OR5K1 : $-CH_3 < -CH_2-CH_3 < < -OCH_3$

OR2AG1: $-CH_3 < -CH_2-CH_3 < < -OCH_3$

OR5K1 : -H > -CH3

OR2AG1 : -H



OR5K1 : -H < -CH3

OR2AG1 : -H

Steric hindrance: ↓ **OR5K1** ↑ **OR2AG1**

OR5K1 : $-H < -CH_3 = -CH_2-CH_3 > -CH_2-CH(CH_3)_2$

OR2AG1: $-CH_2-CH_3 < -CH(CH_3)_2 = -CH_2-CH(CH_3)_2$

Substitution by $-CH_3$ in R3 or R4 is detrimental for **OR2AG1**

Substitution in R1 and R2 is mandatory for **OR2AG1**

Figure 3.- Olfactophore of pyrazine agonists of OR5K1 and OR2AG1. The ranking of molecular features which are necessary for the activation of the receptor are based on the potency ($logEC_{50}$) and the efficacy (E_{max}) obtained in concentration-response experiments. The involvement of R3 and R4 substitutions for the activity of OR5K1 remains to be further investigated.
*HBA = H bond acceptor.

Introduction:

ChemCom progresses towards its objective of deorphanizing the whole repertoire of human olfactory receptors (hORs). Relying on (i) its proprietary technology, (ii) libraries of thousands of odorant compounds and (iii) an efficient screening system, ChemCom is currently identifying and characterizing new modulating molecules (enhancers or blockers) and novel odorant compounds for the whole range of hORs. At ChemCom, more than 120 hORs have been robustly deorphanized.

Here, we present a comparative structure-activity relationship (SAR) data for two unrelated hORs, OR5K1 and OR2AG1, highly expressed in the whole olfactory mucosa[1]. Their most potent agonists were found among pyrazines, with an EC_{50} below the micromolar range.

Pyrazines have a main olfactory note generally qualified as "nutty" or "green", and are key compounds of french fries[2], roasted beef[3], cigar smoker's breath[4], coffee[5], sunless tanning agents[6] odors. The understanding of the molecular mechanisms that support the perception of pyrazine derivatives would certainly help ChemCom to find relevant pharmacomodulators for reducing these malodors.

Methods:

Deorphanization screening was performed in HEK293T-hRTP1S/hRTP2 cells using the CRE-Luciferase reporter assay system[7]. Briefly, cells plated one day before, were transfected with OR and pGL4.29 plasmids using TransIT®-LT1 (Mirus) according to the manufacturer's protocol. Twenty hours after transfection and four hours after incubation with tested compounds, cells were lysed and processed for luminescence measurement using a Spectra Max M5 reader (Molecular Devices).

The compounds of the screening library are distributed in 96 well plates (80 compounds per plate). Each molecule of the library is tested at 316 µM, 100 µM and 31.6 µM. Hits are defined as compounds eliciting a Luciferase response over the plate median value + 2 standard deviations for at least two concentrations. Hits are further validated by concentration-response analyses using the same functional assay. Before being considered as true agonist of the tested OR, validated hits are assessed on mock-transfected cells to confirm the specific activation of the receptor.

Structure-activity relationship studies that compare the activity of different agonists on OR5K1 and OR2AG1 were performed using the Luciferase assay. Results of concentration-response analyses are expressed as the percentage of the response induced by 10 µM of Forskolin (FSK) and were fitted to the Hill's equation.

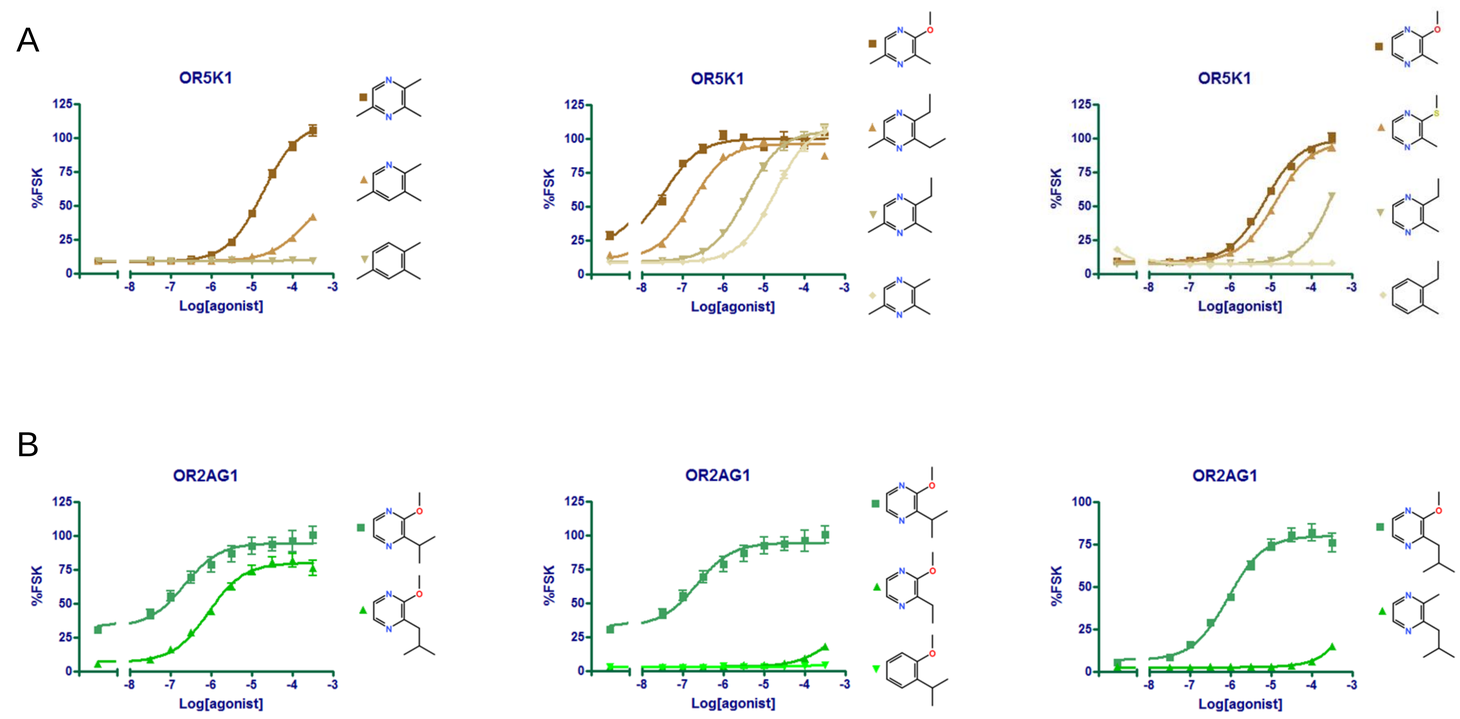


Figure 2.- Concentration-response analysis of representative pyrazine agonists of OR5K1 (A) and OR2AG1 (B) tested in SAR study. Comparison of Luciferase activities of pyridine, benzene and other derivatives.

OR5K1 and OR2AG1 respond to pyrazines molecules:

The two receptors have been included in the human OR deorphanization program that aims to identify odorant activators for the pool of the 273 potentially functional human ORs[1]. During the different screening campaigns performed in the framework of this program, a total of 1100 compounds have been tested. From these screenings, 2,5-dimethylpyrazine (Table 1, **20**) was identified as a specific activator of OR5K1 while 2-isopropyl-3-methoxypyrazine (**1**) was found to trigger OR2AG1.

To further explore this hypothesis, a series of 80 compounds among which linear aliphatic amines, cyclic aliphatic amines, aromatic amines, pyrazine, pyridine, indole and benzene derivatives were tested as potential agonists of the receptors in concentration-response experiments. This study confirmed that OR5K1 and OR2AG1 are triggered by members of the pyrazine family (Table 1). The SAR study revealed that OR5K1 presents a wider selectivity than OR2AG1. None of the tested molecules is an exclusive ligand of OR2AG1. The most potent agonists, having an EC_{50} below the micromolar range, were found among the pyrazines, with 2-methoxy-3,5-dimethylpyrazine (**13**) and 2-isopropyl-3-methoxypyrazine (**1**) as best activators of OR5K1 and OR2AG1, respectively. For OR5K1, the two nitrogen atoms and the two ethyl moieties make the agonist more potent. The benzene derivatives are inactive. A methoxy or a methylthio function replacing one of the ethyls increases the activity of the ligand (Figure 2A). For OR2AG1, the isopropyl or isobutyl group, the 2 nitrogen atoms and the methoxy function are necessary for the full activity of the receptor (Figure 2B). The unsubstituted pyrazine is inactive. Substitution in R1 (for OR5K1) and in R1 and R2 (for OR2AG1) are mandatory. Substitution by a methyl group in R3 or R4 is detrimental for OR2AG1, but increases the activity of OR5K1 (Figure 3).

From the organoleptic point of view, agonists of OR2AG1 are often described as "green, pea, earthy, beany" whereas OR5K1 ligands include more often "nutty, musty, roasted, almond, hazelnut, peanut" characters (Table 1).

In the literature, OR5K1 and OR2AG1 were reported to respond to eugenol methyl ether[8] and to amyl butyrate[9], respectively. Although we succeeded to replicate the results obtained on OR5K1, we did not observe any amyl butyrate activity on OR2AG1 using the Luciferase based gene reporter assay. On another hand, the agonist status of pyrazines was confirmed by other laboratories[10-11].

Many applications : air cleaner, breath spray or tanning products composed by hORs antagonists of OR5K1 and OR2AG1 :

- 2-ethyl-3,5-dimethylpyrazine (**9**), 3-ethyl-2,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine (**14**) and 3-isobutyl-2-methoxypyrazine (**3**) are key compounds of odor of french fries prepared in palm oil[2].
- 2-ethyl-3,5-dimethylpyrazine (**9**) and 2,3-diethyl-5-methylpyrazine (**14**) are key compounds of roasted beef odor[3].
- 2,3-dimethylpyrazine and 2-ethylpyridine are components extracted from the surface of the tongue following smoking of a cigar and 2,3,5-trimethylpyridine, 2,5-dimethylpyrazine (**20**), and 2-ethyl-3,5-dimethylpyridine were identified from the headspace of an aqueous simulated saliva solution treated with cigar smoke[4].
- Several alkyl pyrazines had the greatest impact on the coffee flavor[5].
- Traditional sunless tanning products contain dihydroxyacetone (DHA) as the active ingredient. DHA reacts with the amino groups of the proteins in the stratum corneum in a Maillard reaction to produce pigments called melanoidins. Dimethylpyrazines (**16**, **20**), eliciting an unpleasant odor[6], are also generated by this reaction.

Discussion:

In this study, we demonstrated that pyrazine molecules are the most potent activators of two unrelated hORs, OR5K1 and OR2AG1, which share only 26% sequence identity. This is reflected by the recorded EC_{50} below the micromolar range that 100 times outclasses the previously reported responses of these two ORs.

In addition to their demonstrated expression in the whole olfactory mucosa[1], our results are strongly supportive of an implication of these hORs in the olfactory perception of pyrazines eliciting "nutty" and "green" odors. Even if our results tend to show an preferential association between OR5K1 and the "nutty" note, whereas both OR5K1 and OR2AG1 are associated with the "green" character, we cannot rule out the possibility that each OR plays a role in the perception both the "nutty" and the "green" hues of the tested odorants. The olfactory perception of these odors probably relies on more complex interactions since other receptors, not mentioned in this study, were also identified at ChemCom as pyrazine-selective receptors.

The identification of different antagonists acting selectively on OR5K1 or OR2AG1 and their evaluation as odor blockers will help to clarify the implication of each hORs in the perception of pyrazines. Moreover, as some of the key components of french fries and roasted beef odors correspond to the best agonists identified so far for both ORs, we may predict that the identification of such antagonists will lead to the formulation of new air cleaners, more effective in reducing the corresponding malodors.

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