Identification of antagonists of OR7D4 that neutralize the perception of androstenone

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Introduction :

Olfactory receptors (OR) constitute the largest family of G protein-coupled receptors and are dedicated to the perception of odorant compounds. The development of OR functional assays based on heterologous expression systems has paved the way for the identification of ligands of these sensory receptors, encompassing agonists and antagonists as well as positive and negative allosteric modulators.

The search of antagonist, well developed for receptors of pharmaceutical interest, can be transposed to OR for identifying volatile compounds that would suppressed the activity of a given OR and hence would modify the perception of an odorant molecule that triggers this receptor. So far, there are few reliable experimental demonstrations of this concept. Here, we present the first reported discovery of antagonists of the well-studied receptor OR7D4 that plays a central role in the perception of the male sweat compounds androstenone (AND) and androstadienone.

1. Screening of antagonists :

ChemCom has set up a Luciferase-based gene reporter assay platform that allows to monitor the activation and inhibition of vertebrates OR, including humans. This platform has been used to screen different antagonist libraries, totalizing 4000 compounds, on OR7D4 stimulated by AND. In one of these libraries, encompassing 80 compounds, we identified the ethyl 4-benzylidene-2-methyldec-2-enoate (hereafter referred as compound **1**) as a specific and potent antagonist of OR7D4. Indeed, 316 µM and 100 µM of compound **1** reduced by more that 50% the production of Luciferase induced by AND receptor activation, but remained without any effect on the response induced by forskolin (FSK), a receptor-independent inducer of Luciferase production.

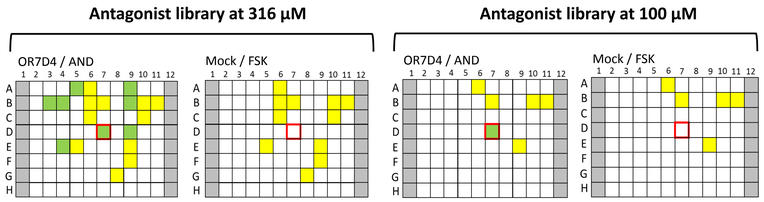

Figure 1. Summary of the inhibition pattern obtained with a sublibrary of 80 compounds on OR7D4.
*ChemCom's modified HEK293 cells were transfected with either OR7D4 or an empty vector (Mock). They were stimulated by androstenone (AND) or forskolin (FSK) as indicated, in the presence of antagonist at either 316 µM or 100 µM, as indicated. Each square of the grid corresponds to one of the 80 compounds of the sublibrary set in a 96 wells plate. Green squares correspond to antagonist compounds that have produced a 50% or more reduction of the response induced by 10 µM androstenone (AND) on OR7D4. Yellow squares correspond to compounds that have produced a 50% or more reduction of the response induced by either AND or FSK and therefore considered as non specific inhibitors. White squares, no inhibition. Grey squares correspond to plate controls. Position of compound **1** is indicated by a red overlining.*

2. Characterization of the discovered antagonist :

The antagonistic effect of compound 1 was further characterized by concentration-response experiments using the Luciferase-based assay. It was first shown that compound **1** inhibits the response of OR7D4 elicited by either AND or its analog androstadienone, the 2 known activators of the receptor (fig. 2 A).

The specific interaction of compound **1** with OR7D4 was further confirmed by assessing its effect on the beta-adrenergic receptor endogenously expressed in HEK293 and on the olfactory receptor OR51E1 (fig. 2 B). As observed for forskolin, compound **1** did not produced a robust inhibition of these receptors.

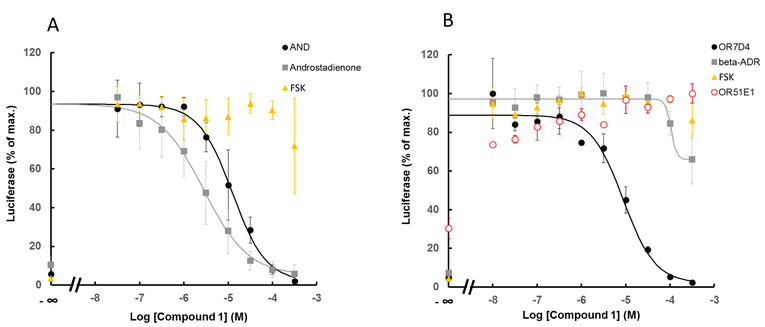

*Figure 2. Confirmation of the specific inhibitory effect of compound **1** on OR7D4.*
*A. HEK293 cells were transfected with OR7D4 and stimulated with either 31.6 µM of androstenone (AND) or androstadienone or 10 µM of FSK, in the presence of indicated concentrations of compound **1**.*
*B. HEK293 cells were transfected with OR7D4, OR51E1 or an empty vector and stimulated with 31.6 µM of AND (OR7D4), 100 µM of isovaleric acid (OR51E1), 10 µM of the beta-adrenergic receptor (beta-ADR) agonist isoproterenol (empty vector) or FSK (empty vector) in the presence of the indicated concentrations of compound **1**.*
Results are the average ± SD of 4 measurements from 2 independent experiments.

To complete the characterization, the influence of a fixed dose of the antagonist on the concentration-response elicited by AND on OR7D4 was explored (fig. 3).

As expected, the EC50 of AND concentration-response curve is shifted to higher values in presence of growing concentrations of compound **1**. The E max values seem to be also reduced suggesting that compound **1** behaves as a non competitive antagonist. This hypothesis requires to be confirmed by an alternative functional assay.

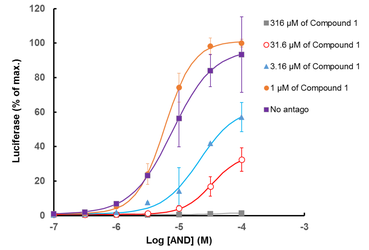

*Figure 3. Influence of compound **1** on AND elicited concentration response.*
*HEK293 cells were transfected with OR7D4 and stimulated with growing concentrations of AND in the presence of indicated single concentrations of compound **1**.*

3. Structure-activity relationship study :

A structure-activity relationship study was performed in a homogenous experiment to determine the optimal antagonist scaffold, based on the structure of compound **1**. It was first observed that suppressing the methyl inserted on the second carbon of the aliphatic chain (fig 4, n°**2**) does not influence drastically the efficiency of the antagonist. Likewise, a reduction of 1 carbon in the main chain (n°**3**) has no effect on the activity, whereas removing more than 3 carbons strongly reduces (n°**4**) or kills (n°**5**) the antagonist potency. A cyclisation of the terminal part of the chain (n°**6**) still keeps a good antagonist activity suggesting that this moiety provides an hydrophobic bulk important for the inhibitory activity. If this bulk is positioned on the aromatic ring (n°**7**), it is less efficient.

Substitution of the ester moiety of n°**1** by a ketone (n°**8**) or an amide (n°**9** and **10**) maintains a strong antagonist activity, whereas nitrile (n°**11**) and alcohol (n°**12**) perform less well. A more drastic reduction of activity is recorded for acid (n°**13**) and ether (n°**14**).

Finally, substitutions in the para position of the aromatic ring with a fluor (n°**15**) or a nitrile (n°**16**) results in a decreased potency of the antagonist.

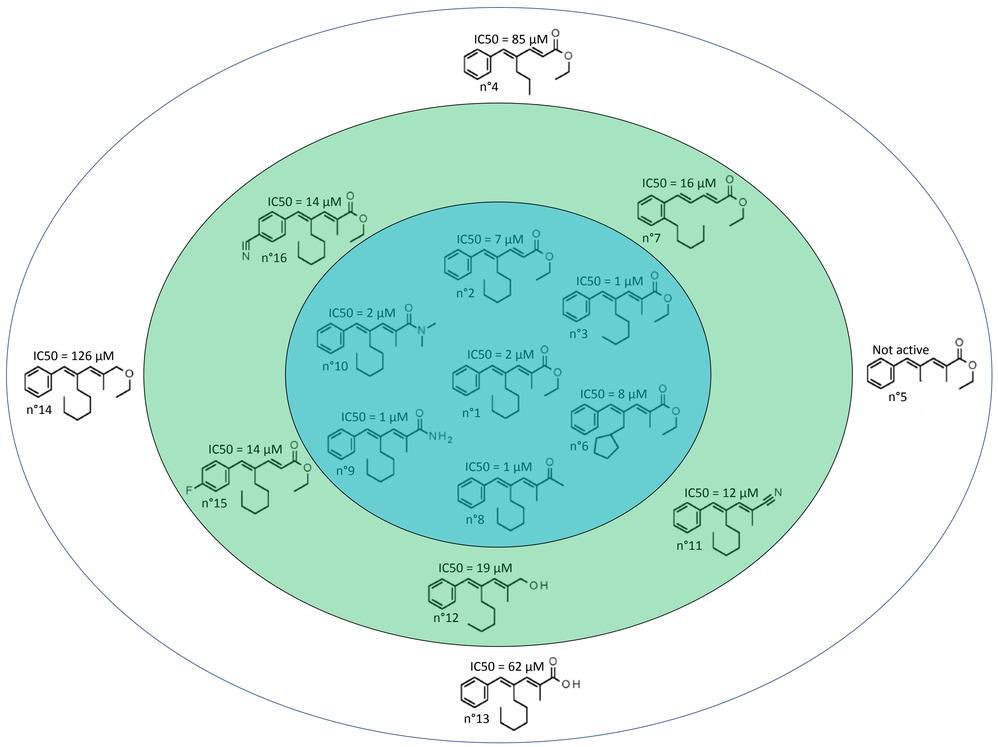

Figure 4: Evaluation of the antagonist potential of molecules structurally related to compound 1.
The represented compounds were synthesized and assessed as potential antagonists of OR7D4 by concentration-response experiments as described in figure 2. Compounds disclosing the lowest IC50 (below 10 µM) are in the blue area, those with an IC50 between 10 and 20 µM are in the green area and those with an IC50 over 50 µM are in the white area

4. Sensory assessment of OR7D4 antagonist :

It was previously shown that OR7D4 plays a central role in the perception of AND by humans (Keller et al., 2007 *Nature* doi:10.1038). Therefore, strong antagonists of this receptor should act as inhibitors of the smell of AND. To test this hypothesis, a first evaluation using a "triangle test" was performed with compound **1** and compound **8** (fig. 5). It consists, for panelists able to smell AND, to evaluate the odor intensity of this molecule in jars containing AND alone or combined with antagonists. Results show that both compound **1** and compound **8** strongly reduce or completely suppress the odor intensity of AND.

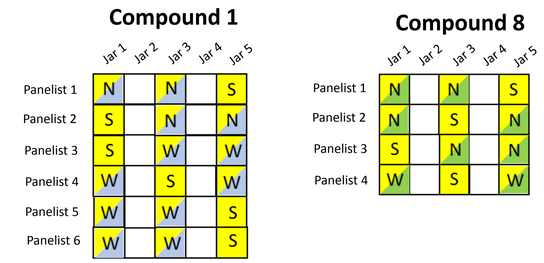

*Figure 5. Evaluation of the blocking potential of compounds **1** and **8** on the perception of AND, using a triangle test.*
A set of 5 jars of 120 ml containing a cotton cloth impregnated with 3 µg of AND alone (yellow squares) or with 200 µl of neat compound 1 (yellow/blue) or compound 8 (yellow/green). White squares correspond to blank jars introduced in the jar set to avoid « carry over » effect of either AND or antagonist compound. Panelists were asked to smell sequentially their set of jars, from 1 to 5 and to assess the intensity of AND odor in jars 1, 3 and 5. « N » indicates no odor, «W » indicates a weak AND odor, « S » indicates a strong AND odor.

A second test (fig. 6), focused on compound **1**, was performed by an independent group of 4 panelists trained to discriminate and to quantify the different odor notes in odor mixtures. In this experiment, AND alone or combined with compound **1** was vaporized in Teldar bags previously filled with a fixed volume of pure air. Panelists were asked to quantify on a scale from 0 (no odor) to 5 (saturating odor) the intensity of each odor note they perceived when releasing air from the bag. According to this experiment, in the presence of compound **1**, there was a complete loss of the characteristic AND odor while a faint odor of mushroom was detected. This odor note is due to the intrinsic smell of compound **1**. Since the odor intensity of the antagonist is clearly weaker than the one of AND, the lack in AND perception can hardly be explained by a covering or masking effect of compound **1**.

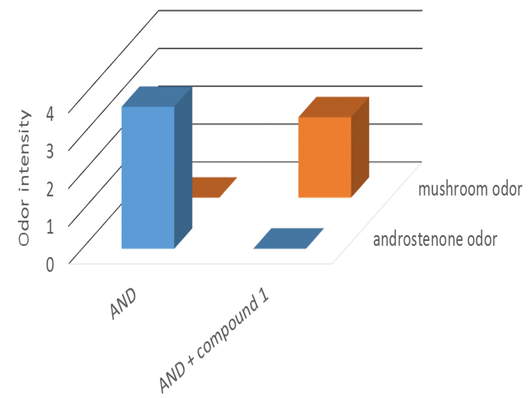

*Figure 6. Confirmation of the blocking potential of compounds **1** on the perception of AND.*

Discussion:

This study discloses a series of so far unidentified antagonists for the olfactory receptor OR7D4 that mediates the perception of androstenone, a steroid compound present in human sweat but also in urine and male pork meat. Among these inhibitors, some possess a strong potency, with an IC50 in the micromolar range that makes them good candidates as neutralizers of the characteristic androstenone smell, generally described as unpleasant by strong smellers. A second interesting characteristic of some of these antagonists is there low intrinsic odor that is compatible with their use in products such as body deodorants or air cleaners. At least two of these compounds were shown to efficiently reduce the perception of androstenone and could be useful for that kind of applications. The whole series of compounds have been patented (Chatelain et al., *« Compounds reducing Malodour perception and the use thereof »* **WO2018/138369**).

Beyond the identification of efficient neutralizers of the malodor of a sweat compounds, our study demonstrates the feasibility of the in vitro approach for finding of new modulators of olfactory perception.

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