nature portfolio

Peer Review File

A bitter anti-inflammatory drug binds at two distinct sites of a human bitter taste GPCR

Corresponding Author: Professor Masha Niv

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript shows Cryo-EM structure of bitter taste receptor (TAS2R14) complexed with FFA ligands and gustducin complex. While the structure of TASR14 complexed with bitter chemical was scooped in Nature this year, the present structure first displays that two FFA ligands bind to canonical orthosteric pocket and intracellular pocket, in contrast to the previous structure, in which one bitter molecule binds to the same intracellular site but the orthosteric pocket was occupied by cholesterol. The two FFA binding was confirmed by nanoBRET analysis, which also showed that two copies have similar affinities. Extensive mutant analyses revealed that mutations of canonical binding pocket have modest effect for receptor activation: unexpectedly, even mutations of Trp3.32 acting in class-A toggle switch and NPxxY activation motif had very small reduction in TAS2R14 activation. In contrast, mutations in the second intracellular binding site largely affected the GPCR activation: especially breakage of His7.49-Arg5.50 hydrogen-bond completely damaged the receptor activation. Therefore, the authors named the His-Arg bond as microswitch. Then, the authors compared TAS2R14 with other receptors and discussed the intracellular pocket with other reported intracellular ligands. This paper is the first to confirm the two copies of agonistic ligands bind to the canonical and second intracellular sites with similar affinities and to speculate the second site acts in the real GPCR activation pathway. Therefore, this reviewer thinks the contribution is significant and should be published in Nature Communications once the authors properly address the following concerns.

1. Why in the previous Nature paper the agonistic ligand only binds to the second intracellular site, while in this paper FFA binds to two sites ?

2. The experimental results that mutations of canonical orthosteric pocket, toggle switch and class-A activation motif showed minimal effect are very impressive. Can the authors more explicitly describe the biological significance of the second intracellular binding pocket ?

3. In the case of PTH1R complexed with biased agonist, PCO371, mutations in the canonical activation pathway had no effect on GPCR activation. It was reported in class-B GPCR, the interface between GPCR and G-protein is occupied two lipid molecules, which was suggested to be related to GPCR basal activity. In the case of TAS2R14, can the authors add some discussion on biological meaning of the intracellular sites in terms of lipid binding and basal activity ?

4. In the second intracellular site, Arg5.50-His7.49 hydrogen bond was shown to be very important for the receptor activation, referring to a microswitch. The authors should more explicitly explain the TAS2R14 activation mechanism triggered by the second intracellular binding. How the structural change is propagated through this Arg5.50-His7.49 hydrogen bond ?

5. In the canonical orthosteric site, the bending of TM7 at Pro7.46 was shown to be important for ligand selectivity. Then, mutation of Pro7.46 affects ligand specificity ?

Reviewer #2

(Remarks to the Author)
Bitter taste is detected through the activation of bitter taste receptors called TAS2Rs. Humans possess 25 different TAS2Rs used to detect hundreds of bitter compounds. While belonging to the GPCR superfamily, TAS2Rs show low sequence identity with any GPCRs. The structural elucidation of bitter taste receptors has been a challenge for years, but it is currently

greatly and quickly advancing. Especially, the first three structures of TAS2R14 were published (Kim et al. and Hu et al.) or made available as a preprint (the present manuscript) in the past two months. In “A bitter anti-inflammatory drug bonds at two distinct sites of a human bitter taste GPCR”, Peri, Matzov, Huxley, et al. provide a structural elucidation by cryo-EM of the bitter receptor TAS2R14 bound to fulfenamic acid (FFA) and an engineered version of the alpha subunit of G-gustducin. Therefore, this manuscript contributes to our new understanding of the bitter taste receptor structures. The same complex TAS2R14/FFA has been part of the structural elucidation effort published by Hu et al. where they observed, as in the present manuscript, the presence of two main binding sites at the intracellular and extracellular sides of the receptor. However, as in Kim et al., the authors identified cholesterol at the intracellular binding site whereas Peri, Matzov, Huxley, et al observed the presence of a second molecule of FFA. This last point is in contradiction with the published structures of TAS2R14, including TAS2R14 bound to FFA. The demonstration of this alternative binding of two molecules of FFA should be made stronger in this manuscript. Hopefully, the comments listed below will help.
The absence of cholesterol in the extracellular binding cavity needs to be clearly demonstrated. The density maps from Kim et al. and Hu et al. should be superimposed with the one from this manuscript, especially the cholesterol/FFA part. Authors should also try to fit cholesterol in the extracellular ligand density map to show the impossibility of its presence in their structure.
In the presented version of the manuscript, the assay proving the binding of FFA at the extracellular side is the NanoBRET-based assay with the TP46 molecule and the intracellular or extracellular Nluc-labeled TAS2R14. This assay is a real plus and could be used to answer concerns.
It would be helpful to dock TP46 in their TAS2R14 structure on both sites to show a prediction/possibility of binding and any eventual difference of the FFA derivative orientation in comparison to the TAS2R14/FFA cryo-EM structure.
Furthermore, the authors' mutation screening of the extracellular or intracellular binding sites is not allowing any conclusion about the stabilization mode of FFA at these sites as all mutants are showing low to no effect on TAS2R14 response. The only mutant showing a significant loss of functon(the way this significance has been determined needs to be described) is at residue G283, which is not interacting with FFA but probably more allowing space for the molecule. Authors suggest that the FFA at the second intracellular site compensates for any loss of binding at the extracellular site. To show that point, it seems necessary to perform TP46 NanoBRET assays from Figure 2 with every mutated version of TAS2R14 to prove that extracellular or intracellular mutations affect the intra or extracellular site binding specifically. It would also help to demonstrate the binding of FFA derivative at the extracellular site.
Out of this mutation screening, authors show the role of a microswitch composed of R2x50 and H7x49 in TAS2R14 activation mechanism, which brings a new piece of knowledge on TAS2R14 activation mechanism.
In a very large part of the manuscript, the authors compare their TAS2R14 structure to the structure of TAS2R46. The authors should make a similar effort devoted to the comparison of their TAS2R14 structure with the two other cryo-EM structures available for this receptor. Part of it should include a discussion identifying any differences in methodology between this study and the previously published work that could explain such different conclusions.
Within this part, the authors notice a displacement of the intracellular part of TM6, allowing the binding of FFA intracellularly. They point at a difference of residue between TAS2R14 and TAS2R46 at the position 7x50. It is very unclear how this position could influence the position of TM6 and the possibility of an intracellular binding site.
The authors finish with a comparison of several GPCR structures showing the binding of a ligand at their intracellular site. They state that the main difference between TAS2R14 and these GPCR structures is that TAS2R14 also binds to a ligand in its extracellular binding site. The authors should also discuss the obvious difference which is that, for the vast majority of these GPCRs, only antagonists are binding to this intracellular site, serving the role of blocking the binding of G proteins.
Here are a few minor points:
BW numbering and label of the TMs should be added in Figures 3 and 4
P7x46, position 7x50, 6x27 and 6x30 should be represented in Figure 5
Reference 56 needs to be corrected.

Reviewer #3

(Remarks to the Author)
Overall, I found this a high quality and interesting MS to read. My expertise is more chemical than biological, so I have concentrated on chemical aspects more.
Do you think binding of FFA to each of the binding sites coupled, ie binding at one site allosterically influences binding of the other? How could one test this? Maybe using different types of BRET sensors for extra versus intra binding sites? (I am not suggesting this is addressed in this MS but wonder if some speculations could be made from any of the results?)
It would be interesting to test the fluorescent probes in whole cell assays rather than membrane preparations. The agonist-induced internalisation of TAS2R14 is not discussed, in particular the time scale (hours?) for this to occur.

Minor comments / potential corrections

- In the introduction, it is stated that FFA is a prescription NSAID…. This statement is a bit misleading as in most countries this drug is not used/prescribed/even available because of side-effects. Mefenamic acid is structurally similar and much more widely used.
- The last few sentences of the introduction reads more like an abstract, not an introduction.
- The minus charge on the CO2- in figure 1b is very small and hard to see.
- Any time a new fluorescent probe is developed it is good practice to characterise pharmacologically as a new chemical entity. It would be useful to know if TP46 retains the same ligand functon(agonist, partial agonist?) as FFA. This information is not required for the BRET experiment results to be analysed but would add to the MS.
- The study by Kim et al, top of page 6, the presence of cholesterol in the orthosteric site in their study – could this come

down to a concentration of cholesterol in the experiment? Ie could cholesterol occupy the orthosteric site in their study, and not yours, as they had a higher concentration of cholesterol? Is the cholesterol concentration comparable between studies? Would a higher concentration of cholesterol prevent or wash out FFA binding in the orthosteric site in your study?

Supporting info corrections

- There are several places in the 1H NMR data that list “0H” as the number of protons. Please carry out a “find” for this, I saw 2 (line 361, line 515). If this is a solvent or impurity, then explain this.
- were 13C NMR spectra run with proton-decoupling? A mixture of decoupled and not? throughout the SI, there is inconsistent occurrences of some carbons listed as, eg, doublet and a J value given, but not for all data and no reasons given, the general expt methods do not outline what experiment was run and if this varied. Some 13C is also reported as a range, eg line 605 “70.88 – 68.90 (m)” this is not usual for reporting 13C data as the reader does not know how many carbons are within this range. Please explain.
- LW380, line 400, unfinished sentence “. Mo “
- the TAMRA-containing compounds, using LW386 as an example, the molecular formula of the exact structure as drawn is C49H51N7O7, so how can the calculated [M+H]+ in the HRMS be the same formula? Where is the “+H” come from? And why is it not H52, as this is the structure drawn plus one hydrogen. Is this all meant to be [M]+ ?
- LW389, line 508, 1H NMR data has many more protons listed than is in the structure.
- using LW389 as an example – is this a novel compound? the reader can not tell easily. And there is no 13C or HRMS. This is standard for novel compounds. If 13C has not be done for final TAMRA-containing compounds due to not enough sample
- then state/justify this somewhere.
- some of the HRMS calculated and found differences are greater than being within acceptable ppm.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this revision, the authors explicitly address my concerns in detail and by a new functional data. Therefore, I now recommend publication of this manuscript in Nature Communications.

Reviewer #2

(Remarks to the Author)
Thank you to the authors for their response and the clarifications provided in the manuscript. There are no further critical points in this study.
The manuscript represents a significant advancement in the molecular understanding of bitter perception and GPCR function as a whole.

Reviewer #3

(Remarks to the Author)
The authors have amended their manuscript in response to my first review comments to a high standard, and I don't have any further comments. I have not reviewed the responses and amendments in detail to the other reviewer reports.

Point-by-Point Reply

We would like to thank all reviewers for their critique of our work, which has enabled us to refine and improve the manuscript. Below we provide a point-by-point response to each of their comments (in blue). The main text changes are shown in blue font in the revised manuscript and in the supplementary information file (for reviewers only). Following newly obtained data or new analysis Fig. 3, Fig. 4 and Fig. 6, as well as Supplementary Fig. 5d-f and Supplementary Fig. 7 were updated and a new Supplementary Fig. 6 was added.

Reviewer #1:

This manuscript shows Cryo-EM structure of bitter taste receptor (TAS2R14) complexed with FFA ligands and gustducin complex. While the structure of TASR14 complexed with bitter chemical was scooped in Nature this year, the present structure first displays that two FFA ligands bind to canonical orthosteric pocket and intracellular pocket, in contrast to the previous structure, in which one bitter molecule binds to the same intracellular site but the orthosteric pocket was occupied by cholesterol. The two FFA binding was confirmed by nanoBRET analysis, which also showed that two copies have similar affinities. Extensive mutant analyses revealed that mutations of canonical binding pocket have modest effect for receptor activation: unexpectedly, even mutations of Trp3.32 acting in class-A toggle switch and NPxxY activation motif had very small reduction in TAS2R14 activation. In contrast, mutations in the second intracellular binding site largely affected the GPCR activation: especially breakage of His7.49-Arg5.50 hydrogen-bond completely damaged the receptor activation. Therefore, the authors named the His-Arg bond as microswitch. Then, the authors compared TAS2R14 with other receptors and discussed the intracellular pocket with other reported intracellular ligands. This paper is the first to confirm the two copies of agonistic ligands bind to the canonical and second intracellular sites with similar affinities and to speculate the second site acts in the real GPCR activation pathway. Therefore, this reviewer thinks the contribution is significant and should be published in Nature Communications once the authors properly address the following concerns.

1. Why in the previous Nature paper the agonistic ligand only binds to the second intracellular site, while in this paper FFA binds to two sites ?

Two studies were recently published in *Nature* while our manuscript was under review, depicting the structure of TAS2R14 in complex with three ligands:

(**1**) a synthetic derivative cmpd28.1, that shares similar scaffold with FFA (*Kim et al., 2024 DOI: 10.1038/s41586-024-07253-y* and *Hu et al. 2024 DOI: 10.1038/s41586-024-07569-9 , both in Nature*); (**2**) FFA (*Hu et al., Nature 2024 DOI: 10.1038/s41586-024-07569-9*) and, (**3**) aristolochic acid (*Hu et al.,Nature 2024 DOI: 10.1038/s41586-024-07569-9*). In these studies, the authors report binding of all three compounds at the intracellular receptor facet, where within the extracellular site, there is a cholesterol molecule. This molecule might originate from purification schemes that include cholesteryl hemisuccinate (CHS) which may be hydrolyzed to cholesterol. These results are in contrast to our current study, where we report two copies of FFA are

simultaneously bound at both the intracellular site of TAS2R14 and within the canonical site of the receptor.

While construct design for the receptor was rather similar between the two studies and our own work, purification schemes differed, especially in regards to detergent and CHS conditions. In the detergent **solubilization** stage, ***Kim et al.*** (DOI: 10.1038/s41586-024-07253-y) used an LMNG/**CHS** mix at 0.6%/**0.12%**, respectively, ***Hu et al.*** (DOI: 10.1038/s41586-024-07569-9) used a 1%/**0.2%** mix, while **our group** used an 0.5%/**0.05%** ratio. In these regards the ***CHS levels were 2.5-4 times higher compared with our purification conditions***. CHS concentrations were also higher in the final SEC buffer, with 0.00015% for the two published papers compared with 0.0001% in our own work.

The higher cholesterol concentration in the two published studies in *Nature* compared with our work may be the reason for the cholesterol density the other groups find in the canonical binding site. In our new supplemental ***Fig. S6a-d*** *(also see* ***Fig. 1*** *below)*, we provide a thorough comparison between the EM densities in the canonical sites obtained in our studies *vs.* the recent studies published in *Nature*. ***These are highlighting that the density recorded for cholesterol does not overlap with FFA density observed in our work, and also that FFA geometry does not fit to the cholesterol density reported by the other two groups.*** For simplicity, we show below only the comparison with the *Kim et al.* structure (PDB 8VY9; EMDB 43650). Similar observations regarding cholesterol position were also reported by *Hu et al*. (DOI:10.1038/s41586-024-07569-9).
In the revised version of the supplementary information, we provide an extended version of this figure (**Fig. S6**) and refer to it in the main text in page 6, lines 1-24.

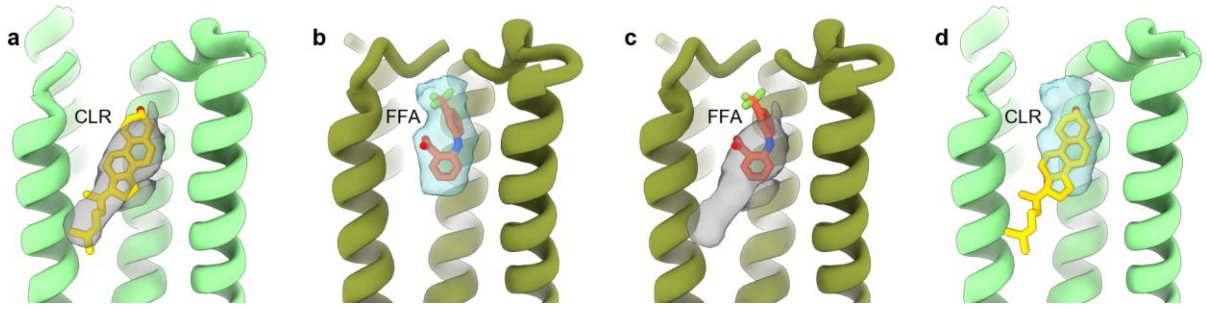

Fig. 1 (S6a-d, in revised version) Comparison of EM densities and models obtained by *Kim et al.* and in the present study. (**a**) Cholesterol (CLR, yellow) in EM density (EMDB: 43650, PDB: 8VY9). (**b**) FFA (orange) in EM density (present study). (**c**) FFA in EMDB: 43650 (*Kim et al.* density), indicating that FFA localization and shape do not correspond to the density observed in *Kim et al.* (gray). (**d**) Cholesterol in the EM density obtained in the current study, indicating that cholesterol position does not overlap with the EM density position and shape. Structures were superposed based on all atom alignment, with RMSD values of 0.769Å.

To support our observations regarding the dual binding mode of FFA to TAS2R14 within the structural studies, we provide ***in our originally submitted manuscript a newly developed NanoBRET assay that further indicates that FFA can bind in both binding pockets***. In this assay we synthesized an FFA mimic (TP46) that is conjugated to a TAMRA fluorophore via a

flexible linker and measure the BRET signal with a nanoluciferase tagged receptor at either the N (extracellular) or C (intracellular) ends (**main Fig. 2**). Our results show that TP46 can bind both pockets with the same affinity, and that elevated concentrations of FFA can substitute TP46 binding. ***In the revised version, we extend these studies and demonstrate that at a high concentration cholesterol is able to partially displace TP46 from the extracellular binding pocket of TAS2R14 (Fig. S6j,k). In agreement with the previously published cryo-EM structures, these data suggest that cholesterol may be able to engage this pocket, at least when used in high concentration. Conversely, TP46 binding to the intracellular pocket is enhanced in the presence of cholesterol, suggesting synergistic effects of TP46 and cholesterol for intracellular ligand recognition by TAS2R14.*** Please also see our response to point 3 for a detailed description on how this new data was incorporated into the revised manuscript.

Finally, while preparing this revision, another study was published in *Cell Research [Tao et al. 2024 DOI:https://doi.org/10.1038/s41422-024-00995-4]*, by which the authors report binding of the same synthetic derivative cmpd28.1 (previously used by both *Kim et al. DOI:10.1038/s41586-024-07253-y* and *Hu et al. DOI: 10.1038/s41586-024-07569-9, Nature 2024*) to also bind TAS2R14 in two copies, in both the extracellular and the intracellular sites. These results are in accordance with our data for FFA, as mentioned on page 8, lines 20-21 of the revised manuscript.

2. The experimental results that mutations of canonical orthosteric pocket, toggle switch and class-A activation motif showed minimal effect are very impressive. Can the authors more explicitly describe the biological significance of the second intracellular binding pocket ?

Our interpretation is that the intracellular binding pocket can compensate for most of the mutations in the extracellular pocket, based on the mild effects observed. This is consistent with our own results and the results obtained in the other TAS2R14 structural papers, that highlight the intracellular pocket as a major contributor to receptor activation, while the extracellular pocket plays a minor role in receptor activation.
In the revised version we further show this through additional binding measurements using our novel NanoBRET ligand binding assay (**Fig. S6j-k**) and further analysis of the signaling data (**revised main Figures 3d** and **4d**). Please see also our response to point 3 to reviewer #2 for further details.
Taken together, the molecular significance of the intracellular pocket is now established via the combination of signaling and binding assays. The biological significance of this pocket is that it enables TAS2R14 to sense intracellular and membrane-permeating ligands, as we now explicitly mention in the revised manuscript, page 9 lines 3-18.

3. In the case of PTH1R complexed with biased agonist, PCO371, mutations in the canonical activation pathway had no effect on GPCR activation. It was reported in class-B GPCR, the interface between GPCR and G-protein is occupied two lipid molecules, which was suggested to be related to GPCR basal activity. In the case of TAS2R14, can the

authors add some discussion on biological meaning of the intracellular sites in terms of lipid binding and basal activity?

We thank the reviewer for this comment. In the current work, the FFA molecule does not overlap with this lipid site, and instead is overlapping with the position of a palmitoyl moiety in a class B1 (adhesion) GPCR (Ping et al., Nature 2021 DOI: https://doi.org/10.1038/s41586-020-03083-w) where the lipid is covalently attached to the G alpha subunit and serves as a bridge to connect the receptor and the G-proteins. This is shown in **main Fig. 6**, as well as in the supplemental **Fig. S12**.
Following the reviewer's question, we added two new analyses: i) we measured the effect of cholesterol on binding of TP46 in the extracellular and the intracellular sites using our novel NanoBRET ligand binding assay. We observed that while at high concentrations cholesterol can partially displace TP46 from the extracelluar site, no such competition is observed for the intracellular site (**Fig. S6j-k**). Instead, we observed that in the presence of elevated cholesterol concentrations, TP46 binding to the intracellular site increases. This data suggests that while the effects of cholesterol on the extracellular pocket are through direct binding to the pocket (also evidenced by the studies of *Kim et al. DOI: 10.1038/s41586-024-07253-y,* and *Hu et al. DOI: 10.1038/s41586-024-07569-9*), the effects on the secondary pocket are most likely through allostery. ii) Further analyzing our cryo-EM data, we indeed see three densities that could accommodate cholesterol at the interface between the receptor and the detergent micelle (**Fig. S6i**). These are localized in close proximity to TM3 and 4 in the outer leaflet, TMs 2, 3 and 4 at one side of the intracellular face, and TMs 3, 5 and H8 on the other side. Additional external binding positions might also occur at elevating concentrations, and thus contribute alone or in combination, to allosteric modulation of the intracellular site. As lipid/cholesterol binding to GPCRs is known to mediate activation, and in light of our new data regarding cholesterol binding, we now added these discussion points to our revised version (page 6, lines 12-14).

4. In the second intracellular site, Arg5.50-His7.49 hydrogen bond was shown to be very important for the receptor activation, referring to a microswitch. The authors should more explicitly explain the TAS2R14 activation mechanism triggered by the second intracellular binding. How the structural change is propagated through this Arg5.50-His7.49 hydrogen bond?

We thank the reviewer for this comment. We decided to take out the discussion of the Arg2.50 residue and the mechanism of activation/inactivation, which will be studied in more depth in future work.

5. In the canonical orthosteric site, the bending of TM7 at Pro7.46 was shown to be important for ligand selectivity. Then, mutation of Pro7.46 affects ligand specificity?

We thank the reviewer for this question, which allows us to better clarify our hypothesis regarding a conserved proline at position 7.46, that is unique to bitter receptors and odorant receptors. In our main text, we highlight this position as unique for this receptor family, and that this proline is

manifesting a kink, that orients the extracellular opening of TM7. We suggested that the role of this proline might be important for determining the size of the upper pocket, potentially to match with a diverse ligand arsenal. We do not imply that this proline might be related to ligand specificity, or that mutating this residue might induce or impair it. Based on the structures available for the taste receptor family, the proline allows for TM7 mobility. It would indeed be interesting to conduct a follow up study, to test whether mutations in this area could preclude ligand binding, or reduce receptor promiscuity and mobility. These interesting questions could not be addressed by a simple residue replacement, and would require a new experimental design (e.g., labeling of the receptor at various locations to follow TM7 mobility by biophysical methods). Thus, those are way beyond the scope of the current study. For clarity, and saving some space, we now significantly trimmed this part of the manuscript.
The new text is provided below:

"The most notable difference in the extracellular TM arrangement is the TM7 position (**Figs. 5c**, **Supplementary Fig. 11a**). In both receptors TM7 has a kink, that is caused by a hinge originating from a shared proline residue in position 7.46 ($P273^{7.46}$ and $P272^{7.46}$ in TAS2R14 and TAS2R46, respectively, (**Supplementary Fig. 9**). However, the tilt level greatly differs, with only a mild tilt (22°) for TAS2R14, and a 45° tilt in TAS2R46, that profoundly opens the extracellular space of the receptor and contributes to the larger void of the binding pocket (**Figs 5c-e**, **Supplementary Fig. 11a**). Considering the differential binding mode of the two ligands within the pockets, it is tempting to speculate that the tilt level might depend on the ligand's geometry. Also, given that the proline residue ($P^{7.46}$) is conserved in most TAS2Rs (**Supplementary Fig. 9**), but not in other GPCRs, this could represent a common mechanism for bitter taste receptors. In fact, the odorant receptor family is the only other GPCR family with conserved $P^{7.46}$ (**Supplementary Fig. 11b**). This family of receptors also recognizes numerous and diverse ligands, which could further support the role of $P^{7.46}$ for versatility".

Reviewer #2:
Bitter taste is detected through the activation of bitter taste receptors called TAS2Rs. Humans possess 25 different TAS2Rs used to detect hundreds of bitter compounds. While belonging to the GPCR superfamily, TAS2Rs show low sequence identity with any GPCRs. The structural elucidation of bitter taste receptors has been a challenge for years, but it is currently greatly and quickly advancing. Especially, the first three structures of TAS2R14 were published (Kim et al. and Hu et al.) or made available as a preprint (the present manuscript) in the past two months. In "A bitter anti-inflammatory drug bonds at two distinct sites of a human bitter taste GPCR", Peri, Matzov, Huxley, et al. provide a structural elucidation by cryo-EM of the bitter receptor TAS2R14 bound to fulfenamic acid (FFA) and an engineered version of the alpha subunit of G-gustducin. Therefore, this manuscript contributes to our new understanding of the bitter taste receptor structures. The same complex TAS2R14/FFA has been part of the structural elucidation effort published by Hu et al. where they observed, as in the present manuscript, the presence of two main binding sites at the intracellular and extracellular sides of the receptor. However, as in Kim et al., the authors identified cholesterol at the intracellular binding site whereas Peri, Matzov, Huxley, et al observed the presence of a second molecule of FFA. This last point is in contradiction with the published structures of TAS2R14, including TAS2R14 bound to FFA. The demonstration of this alternative binding of two molecules of FFA should be made stronger in this manuscript. Hopefully, the comments listed below will help.

The absence of cholesterol in the extracellular binding cavity needs to be clearly demonstrated. The density maps from Kim et al. and Hu et al. should be superimposed with the one from this manuscript, especially the cholesterol/FFA part. Authors should also try to fit cholesterol in the extracellular ligand density map to show the impossibility of its presence in their structure.

We thank the reviewer for these comments. In our response to reviewer #1, the maps have been superposed to show that the density associated with cholesterol in the ***Kim et al.*** *DOI: 10.1038/s41586-024-07253-y* and ***Hu et al.*** (DOI: 10.1038/s41586-024-07569-9) studies, does not correspond to the FFA density presented in our own study (***Fig. 1 in this letter***). An extended version of this figure, that includes also a comparison with a newer publication (*Tao et al. DOI:https://doi.org/10.1038/s41422-024-00995-4*), which, similarly to us, found two copies of a ligand in TAS2R14, is provided as supplementary information in the revised version enclosed (***Fig. S6***) and referred to in main text on page 6, lines 1-24. Please see also an extended response to this point in our answer to the first concern raised by reviewer #1.

In the presented version of the manuscript, the assay proving the binding of FFA at the extracellular side is the NanoBRET-based assay with the TP46 molecule and the intracellular or extracellular Nluc-labeled TAS2R14. This assay is a real plus and could be used to answer concerns.
It would be helpful to dock TP46 in their TAS2R14 structure on both sites to show a prediction/possibility of binding and any eventual difference of the FFA derivative orientation in comparison to the TAS2R14/FFA cryo-EM structure.

As rightfully suggested by the reviewer, we performed docking of TP46 to the receptor (new supplemental **Fig. S5 e,f** also provided below).

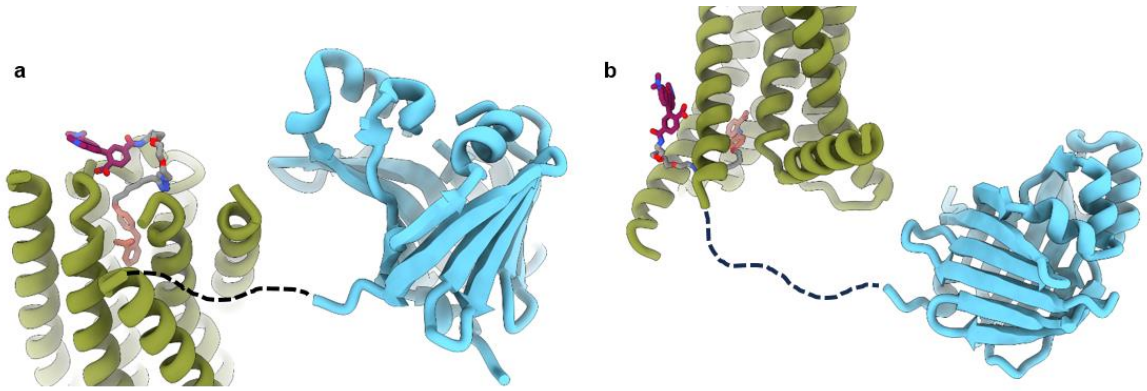

Fig. 2 (S5e,f in revised version) **Docking of TP46 at the two binding pockets with labeled NLuc.** TP46 docked at the (**a**) extracellular and (**b**) intracellular faces, with the FFA scaffold constrained according to poses observed in the cryo-EM structure. The grid was generated based on the ligands of the solved structure. Due to the size of TP46, the maximum size of the grid was selected (dock ligands with length ≤36Å, box size 40Å in x, y, and z axes). TP46 was docked to the receptor using this grid with Glide XP, and core constraints to the FFA (all atoms of the FFA were picked as constraints), restricting the docking to this reference and allowing only ligands that match this pattern to dock. The X-ray structure of NanoLuc

luciferase (PDB ID 5IBO) was placed near the N- and C- terminus of the receptor to illustrate its plausible position.

Furthermore, the authors' mutation screening of the extracellular or intracellular binding sites is not allowing any conclusion about the stabilization mode of FFA at these sites as all mutants are showing low to no effect on TAS2R14 response. The only mutant showing a significant loss of function (the way this significance has been determined needs to be described) is at residue G283, which is not interacting with FFA but probably more allowing space for the molecule. Authors suggest that the FFA at the second intracellular site compensates for any loss of binding at the extracellular site.
To show that point, it seems necessary to perform TP46 NanoBRET assays from Figure 2 with every mutated version of TAS2R14 to prove that extracellular or intracellular mutations affect the intra or extracellular site binding specifically. It would also help to demonstrate the binding of FFA derivative at the extracellular site.

We thank the reviewer for highlighting the fact that the way we were presenting our pharmacological data was somewhat confusing. To improve it and to match the way similar data was presented in the recent TAS2R14 structural papers, we have plotted the change in BRET at a single concentration point in the main text and moved the concentration response curves in the supplemental. This way of presenting the data more clearly highlights those mutations which impact G protein activation and allow for direct comparison with the published studies on the same receptor. The data now more straightforwardly support the importance of the intracellular site for receptor function.

Out of this mutation screening, authors show the role of a microswitch composed of R2x50 and H7x49 in TAS2R14 activation mechanism, which brings a new piece of knowledge on TAS2R14 activation mechanism. ***In a very large part of the manuscript, the authors compare their TAS2R14 structure to the structure of TAS2R46. The authors should make a similar effort devoted to the comparison of their TAS2R14 structure with the two other cryo-EM structures available for this receptor. Part of it should include a discussion identifying any differences in methodology between this study and the previously published work that could explain such different conclusions.***

When we submitted the manuscript, the other structures and most studies were not yet published, thus we only focused our analysis on the available data on TAS2R46. Now that the other studies are publicly available, we have added a full comparison and description to the enclosed revised version (page 6, lines 1-24). Please see also our response to reviewer #1 and **Fig. 1**, for full information.

Within this part, the authors notice a displacement of the intracellular part of TM6, allowing the binding of FFA intracellularly. They point at a difference of residue between TAS2R14 and TAS2R46 at the position 7x50. It is very unclear how this position could influence the position of TM6 and the possibility of an intracellular binding site.

We thank the reviewer for this comment. While we mention the difference between TAS2R14 and TAS2R46 at position 7.50, we now refrain from suggesting that this affects TMs positioning.

The authors finish with a comparison of several GPCR structures showing the binding of a ligand at their intracellular site. They state that the main difference between TAS2R14 and these GPCR structures is that TAS2R14 also binds to a ligand in its extracellular binding site. The authors should also discuss the obvious difference which is that, for the vast majority of these GPCRs, only antagonists are binding to this intracellular site, serving the role of blocking the binding of G proteins.

Intracellular ligands reported so far have been shown to modulate GPCR activation in various ways. Those include agonists, antagonists, inverse agonists, and biased agonists (**main Figure 6**). Similar to the current study, and other recent findings regarding TAS2R14 agonists, the binding of a PTH1R intracellular ligand (PCO371) facilitates receptor agonism. In contrast, for class A chemokine GPCRs, ligands binding the intracellular sites often act as antagonists. The agonists *vs.* antagonist activity of intracellular ligands is highlighted in our **main Figure 6**.

As the ligands reported so far are very limited, it is still difficult to make a conclusion regarding the common/differential features governing their ability to modulate GPCR activity in one way or the other. Also, due to space limitation, in our revised version we only provide a short summary in regards and focus on commonalities and differences to the recently reported structures of TAS2R14 agonists.

Here are a few minor points:

BW numbering and label of the TMs should be added in Figures 3 and 4

The BW numbers were added in both figures.

P7x46, position 7x50, 6x27 and 6x30 should be represented in Figure 5

Positions relevant for the discussions were added in **Figures 5** and **S11**.

Reference 56 needs to be corrected.

The reference has been corrected in the revised version.

Reviewer #3:

Overall, I found this a high quality and interesting MS to read. My expertise is more chemical than biological, so I have concentrated on chemical aspects more.

Do you think binding of FFA to each of the binding sites coupled, ie binding at one site allosterically influences binding of the other? How could one test this? Maybe using different types of BRET sensors for extra versus intra binding sites? (I am not suggesting this is addressed in this MS but wonder if some speculations could be made from any of the results?)

We thank the reviewer for this interesting line of thought. Indeed, allosteric coupling between the canonical extracellular and a similarly located intracellular binding pocket has been shown for other GPCRs (e.g. the Neurotensin receptor subtype 1). In that case, the binding of an intracellular

allosteric modulator (SBI-553) enhances the binding of agonists to the extracellular pocket, which could be demonstrated using radioligand and fluorescent ligand binding studies (Slosky et al. DOI: https://doi.org/10.1016/j.tips.2020.12.005, Vogt et al. DOI:10.1021/acsptsci.4c00086). For TAS2R14, such evidence cannot be as easily collected, as this would require two separate read-outs with specific ligands/probes binding to the intra- and extracellular pockets respectively.

Interestingly, binding experiments conducted with TP46 and cholesterol show an enhancement of TP46 binding to the intracellular pocket in the presence of cholesterol, suggesting an allosteric action of cholesterol on TAS2R14. However, cholesterol only marginally displaced TP46 from the extracellular pocket (new **Fig. S6j,k**), so that no direct conclusions about cholesterol's site of action and the mechanism of the interaction can be drawn. To include this information to the manuscript, we have added the following sentences on page 6.

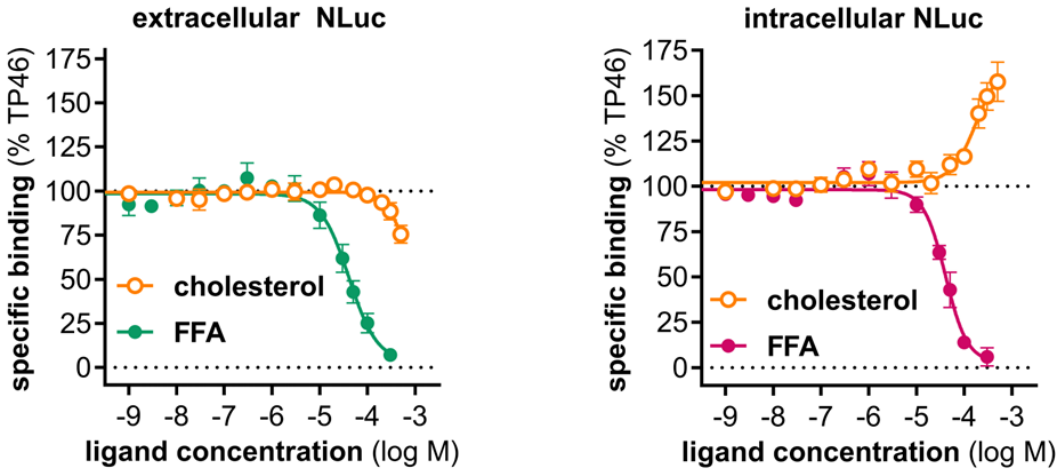

Fig. 3 (S6j,k in revised paper) **Comparison of FFA binding with cholesterol (CLR) and other agonists.** (**j**) Binding experiments with membranes from HEK293T cells expressing TAS2R14 fused to an extracellular nanoluciferase confirm complete displacement of TP46 (1 µM) by FFA and reveal partial displacement of the probe by high concentrations of cholesterol. (**k**) Similar experiments with membranes from HEK293T cells expressing TAS2R14 labeled with an intracellular nanoluciferase show competition of FFA with TP46 (1 µM), while cholesterol enhances TP46 binding. Data show mean ± SEM of n = 5 (FFA) or n = 8 (cholesterol) independent experiments.

It would be interesting to test the fluorescent probes in whole cell assays rather than membrane preparations. The agonist-induced internalisation of TAS2R14 is not discussed, in particular the time scale (hours?) for this to occur.

We agree with the reviewer, that it would be interesting to track ligand binding to TAS2R14 in whole cells. However, this would lead to a less comparable situation regarding the access of the fluorescently labeled probe to the two binding sites. Only the extracellular binding site can be reached by free diffusion in solution while the ligand would have to cross the cell membrane to reach the intracellular compartment of living cells. Although the employed TAMRA fluorophore has been previously shown to be cell permeable, similar nanoBRET binding experiments for the intracellular pockets of the chemokine receptor family revealed slower binding kinetics, higher

non-specific binding and a lower signal to noise ratio (e.g., Toy et al., 2022 DOI:10.1021/acschembio.2c00263) making interpretation of whole cell assays more difficult than ones using membranes.

Agonist-induced phosphorylation by GRKs, the recruitment of β-arrestin and the desensitization of TAS2R14 have been described previously (Kim et al. DOI: 10.1038/s41586-024-07253-y, Woo et al. DOI: 10.1096/fj.201802627RR and Tao et al. DOI: https://doi.org/10.1038/s41422-024-00995-4). In our work we did not study TAS2R14 internalization as we only obtained complexes of the receptor and G proteins, but not GRKs or β-arrestin. It is an interesting idea to use our newly developed fluorescent probe to study TAS2R14 trafficking, but regarding its micromolar affinity, it might be worth further optimizing this molecular probe, with the help of the now available TAS2R14 cryo-EM structures.

Minor comments / potential corrections

- In the introduction, it is stated that FFA is a prescription NSAID…. This statement is a bit misleading as in most countries this drug is not used/prescribed/even available because of side-effects. Mefenamic acid is structurally similar and much more widely used.

We thank the reviewer for pointing this out. In the revised version we changed the statement to the following: "FFA is a nonsteroidal anti-inflammatory **molecule** that acts via inhibition of cyclooxygenase-2, and is also one of the most potent TAS2R14 agonists".

- The last few sentences of the introduction reads more like an abstract, not an introduction.

Per the reviewer's suggestion we have removed the last few introductory sentences from the revised version.

- The minus charge on the CO2- in figure 1b is very small and hard to see.

The minus size for the carboxyl moiety was enlarged.

- Any time a new fluorescent probe is developed it is good practice to characterise pharmacologically as a new chemical entity. It would be useful to know if TP46 retains the same ligand function (agonist, partial agonist?) as FFA. This information is not required for the BRET experiment results to be analysed but would add to the MS.

We agree with the reviewer, it would be interesting to know whether TP46 is able to activate TAS2R14 by binding to the respective receptor pockets. However, TP46's fluorescent properties interfere with the luminescence/fluorescence (BRET, FRET) based read-outs that are established to determine TAS2R14 function, thereby preventing a reliable determination of TP46's activity. To elucidate whether the introduction of the linker to the para-position of FFA's ring B is generally compatible with the activation of TAS2R14, we conducted additional IP1 accumulation assays with TP46's alkyne-precursor LW384. These experiments show that LW384 is a TAS2R14 partial agonist, eliciting approximately 50% of the FFA-mediated TAS2R14 activation (revised **Fig. S5d**). To incorporate this new data, we have added to the manuscript on page 4-5:

"While TP46 could not be investigated for TAS2R14 activation due to its fluorescent nature interfering with the assays' read-outs, the alkyne precursor LW384 was found to be a partial agonist with an E_{max} of ~66% relative to FFA (**Supplementary Fig. 5**), suggesting that the addition of the flexible alkyne linker to the para position of ring B is compatible with G protein coupling to TAS2R14."

The study by Kim et al, top of page 6, the presence of cholesterol in the orthosteric site in their study – could this come down to a concentration of cholesterol in the experiment? Ie could cholesterol occupy the orthosteric site in their study, and not yours, as they had a higher concentration of cholesterol? Is the cholesterol concentration comparable between studies? Would a higher concentration of cholesterol prevent or wash out FFA binding in the orthosteric site in your study?

This is a very valid point, which lead us to conduct binding experiments with cholesterol. Please see above our response to the first bullet point from reviewer #1.

- There are several places in the 1H NMR data that list "0H" as the number of protons. Please carry out a "find" for this, I saw 2 (line 361, line 515). If this is a solvent or impurity, then explain this.

Thank you for spotting this. We removed a residual solvent signal ($CHCl_3$ from $CDCl_3$), that was accidentally included in the peak listings.

- were 13C NMR spectra run with proton-decoupling? A mixture of decoupled and not? throughout the SI, there is inconsistent occurrences of some carbons listed as, eg, doublet and a J value given, but not for all data and no reasons given, the general expt methods do not outline what experiment was run and if this varied. Some 13C is also reported as a range, eg line 605 "70.88 – 68.90 (m)" this is not usual for reporting 13C data as the reader does not know how many carbons are within this range. Please explain.

We apologize for the confusion. All ^{13}C-NMR spectra were recorded as DEPTQ with proton-decoupling. This information was added to the general NMR-spectroscopy section of the Supplementary Information. We reanalyzed all spectra and now report the individual peaks for all signals.

- LW380, line 400, unfinished sentence ". Mo "

We removed the unfinished sentence.

- the TAMRA-containing compounds, using LW386 as an example, the molecular formula of the exact structure as drawn is C49H51N7O7, so how can the calculated [M+H]+ in the HRMS be the same formula? Where is the "+H" come from? And why is it not H52, as this is the structure drawn plus one hydrogen. Is this all meant to be [M]+ ?

All high-resolution mass spectra were recorded in with ESI as the ionization source and molecules were therefore detected as either $[M+H]^+$ or $[(M+2H)/2]^+$. For the mentioned spectrum of LW386, the exact mass (m/z) for $[M+H]^+$ was actually calculated for $C_{49}H_{52}N_7O_7$, but the additional proton was not indicated in the chemical formula to allow simultaneous reporting of $[M+2H]^{2+}$. For

clarification, we now separately report the found/calculated masses for the different ions, including their respective chemical formulas.

- LW389, line 508, 1H NMR data has many more protons listed than is in the structure.

We sincerely apologize for accidentally providing the peak listing of another compound. We reanalyzed the spectrum of LW389 and now provide the correct signals.

- using LW389 as an example – is this a novel compound? the reader can not tell easily. And there is no 13C or HRMS. This is standard for novel compounds. If 13C has not be done for final TAMRA-containing compounds due to not enough sample - then state/justify this somewhere.

Throughout the Supplementary Information, we indicate known compounds with a reference to their previous description (see e.g. LW382). We have added the missing ^{13}C-NMR data for LW389. ^{13}C-NMR data was recorded the majority of the final TAMRA-containing compounds, excluding LW392, L393 and LW394 due to limited amount of sample. HRMS was only recorded for the final TAMRA-containing compounds. To transparently provide this information to the reader, we have added to the general chemistry section on page 7 of the Supplementary Information:

"For the final TAMRA-containing compounds, ESI-TOF high mass accuracy and resolution experiments were performed on […]."

"^{13}C-NMR spectra were not recorded for the fluorescent ligands LW392, LW393 and LW394 due to limited amount of sample".

- some of the HRMS calculated and found differences are greater than being within acceptable ppm.

Thank you for drawing our attention to these inaccuracies. Apparently, the software tool that was initially used to calculate the expected masses resulted in erroneous numbers. We recalculated all exact ionic masses with ChemDraw 23.0 and found that the absolute errors were below 0.0008 (< 1.3 ppm), when comparing the expected and found values for m/z, respectively.