UNIVERSITY OF BIRMINGHAM

University of Birmingham Research at Birmingham

Overexpression of orphan receptor GPR61 increases cAMP levels upon forskolin stimulation in HEK293 cells

Kozielewicz, Pawel; Grafton, Gillian; Sajkowska-Kozielewicz, Joanna J; Barnes, Nicholas M

DOI:
10.1159/000501926

License:
Other (please specify with Rights Statement)

Document Version
Peer reviewed version

Citation for published version (Harvard):
Kozielewicz, P, Grafton, G, Sajkowska-Kozielewicz, JJ & Barnes, NM 2019, 'Overexpression of orphan receptor GPR61 increases cAMP levels upon forskolin stimulation in HEK293 cells: in vitro and in silico validation of 5-(nonyloxy)tryptamine as a low-affinity inverse agonist', *Pharmacology*, vol. 104, no. 5-6, pp. 377-382. https://doi.org/10.1159/000501926

Link to publication on Research at Birmingham portal

Publisher Rights Statement:
This is the peer-reviewed but unedited manuscript version of the following article: Kozielewicz P, Grafton G, Sajkowska-Kozielewicz J, J, Barnes N, M: Overexpression of Orphan Receptor GPR61 Increases cAMP Levels upon Forskolin Stimulation in HEK293 Cells: in vitro and in silico Validation of 5-(Nonyloxy)Tryptamine as a Low-Affinity Inverse Agonist. Pharmacology 2019. DOI: 10.1159/000501926. The final, published version is available at https://www.karger.com/Article/FullText/501926#

General rights
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.
•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
•User may use extracts from the document in line with the concept of ‘fair dealing’ under the Copyright, Designs and Patents Act 1988 (?)
•Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 01. Jun. 2024

Brief Report

Pharmacology

Pharmacology
DOI: 10.1159/000501926

Received: May 27, 2019
Accepted: July 3, 2019
Published online: July 26, 2019

Overexpression of Orphan Receptor GPR61 Increases cAMP Levels upon Forskolin Stimulation in HEK293 Cells: in vitro and in silico Validation of 5-(Nonyloxy) Tryptamine as a Low-Affinity Inverse Agonist

Pawel Kozielewicz[a, b] Gillian Grafton[b] Joanna J. Sajkowska-Kozielewicz[c] Nicholas M. Barnes[b]

[a]Department of Physiology and Pharmacology, Karolinska Insitutet, Solna, Stockholm, Sweden; [b]Institute of Clinical Sciences, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK; [c]Chair of Physical Pharmacy and Bioanalysis, Faculty of Pharmacy, Medical University of Warsaw, Warsaw, Poland

Keywords

GPR61 · Constitutive activity · Inverse agonist · Receptor · Molecular modeling · Proteins

Abstract

GPR61 is an orphan receptor that belongs to Class A of G-protein-coupled receptors. It has been reported that GPR61 has a constitutive activity and couples to Gα_s. In the present study, we characterized GPR61 function and ligand binding by experimental and molecular docking studies. We demonstrated that heterologous expression of GPR61 in HEK293 cells enhanced the cAMP synthesis response to forskolin, whereas the basal cAMP synthesis was unaffected. 5-(Nonyloxy)tryptamine inhibited forskolin-stimulated cAMP production in GPR61-expressing HEK293 cells. These studies highlight that the intrinsic activity of this receptor is only measurable following its synergy with Gα_s. © 2019 S. Karger AG, Basel

Introduction

G-protein-coupled receptors (GPCRs) are the largest family of cell surface receptors. They are also the most common targets (ca. 34%) for FDA-approved drugs [1]. However, despite the arising interest, >150 GPCRs remain "orphan"; that is with no endogenous ligand discovered to date, and 81 of these orphan receptors belong to the Class A GPCRs [2]. GPR61 (other names: GPCR3, Biogenic amine receptor-like G-protein-coupled receptor, BALGR) is an orphan receptor, which sequence was first identified in 2001 [3]. GPR61 is expressed in various tissues including adrenal gland, hippocampus, and leukocytes including proinflammatory Th17 cells [4–6]. Pharmacological targeting of GPR61 should be explored explored in greater detail since, for example, this receptor has been reported to be relevant in metabolism as GPR61-deficient mice developed obesity [7].

KARGER

© 2019 S. Karger AG, Basel

E-Mail karger@karger.com
www.karger.com/pha

Pawel Kozielewicz
Department of Physiology and Pharmacology
Karolinska Insitute
SE–171 65 Solna, Stockholm (Sweden)
E-Mail pawel.kozielewicz@ki.se

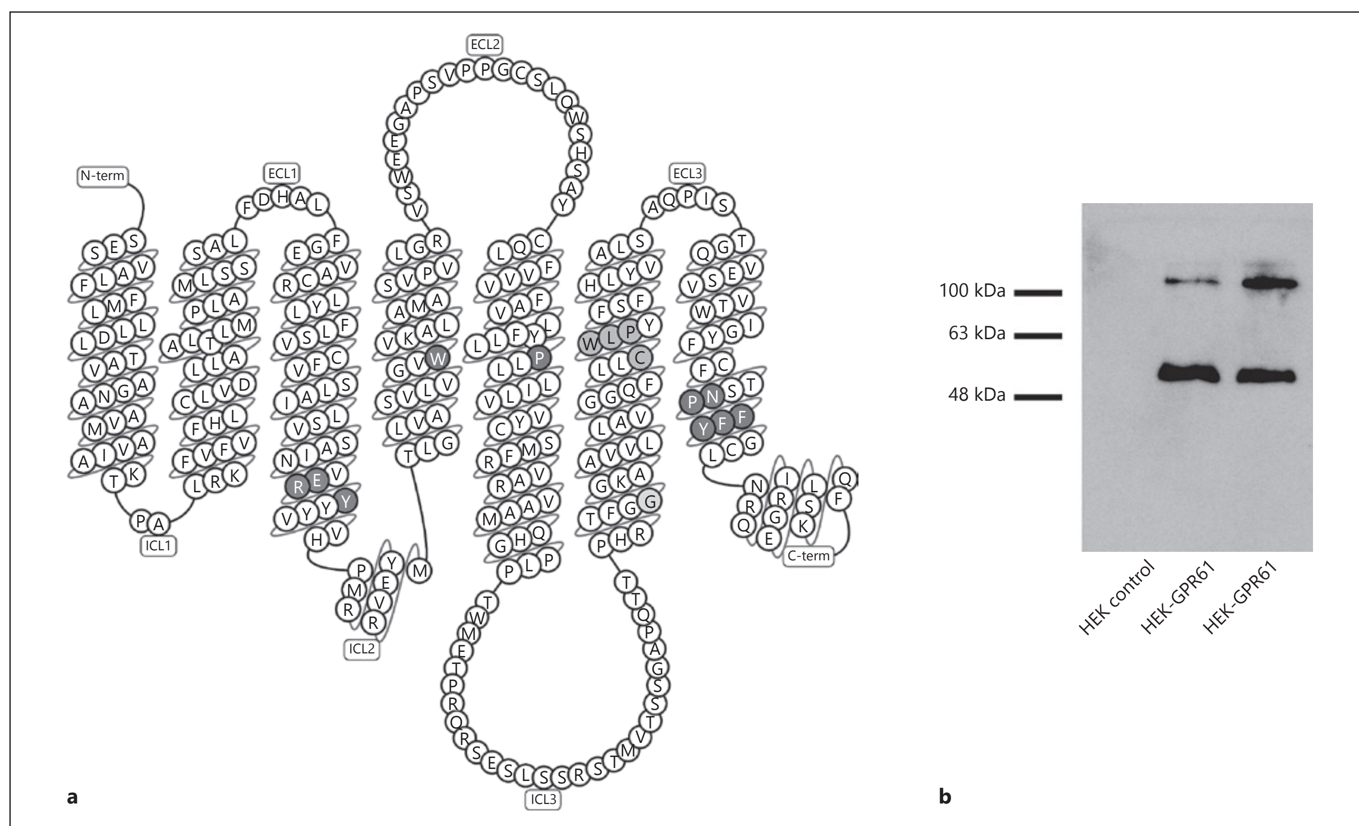

Fig. 1. a GPR61 structure with conserved and important residues/motifs shown highlighted: D(E)RY in TM3; $W^{3.50}$, $P^{4.50}$, CWxP in TM6; and NPxxY in TM7. GPR61 has $G282^{6.30}$ in place of $D^{6.30}$. **b** Myc-immunoreactivity in HEK293 cells stably expressing His-myc-tagged GPR61. GPR61 is expressed both as a monomer and a dimer, Lanes: 1 = HEK293 control cells, 2 and 3 = HEK293 stably overexpressing His-myc-tagged GPR61.

At a structural level, GPR61 has the conserved tyrosine toggle switch NPxxY and CWxP motifs, which are involved in the receptor activation in the Class A GPCRs [8]. However, the receptor lacks the conserved aspartic acid in the transmembrane domain 6 ($D^{6.30}$ in Ballesteros-Weinstein nomenclature system [9]) that forms a classical "ionic lock" with $R^{3.50}$ keeping GPCRs in an inactive state (Fig. 1a) [10]. The lack of this motif suggests a potential constitutive activity of the receptor and in support of this basal activity of a GPR61-Gα_s construct was increased in comparison to other receptors [11]. Furthermore, 5-(nonyloxy)tryptamine (5-[nonyloxy]-1H-indole-3-ethanamine, 5-NOT) has been described as a low-affinity inverse agonist of the GPR61 receptor [10]. A subsequent paper from the same group demonstrated that a minor truncation in the N-terminus of GPR61 and a V19A mutation reduced the constitutive activity in the same GPR61-Gα_s (^{35}S)GTPφS functional model [12]. It was concluded that the N-terminal region of the receptor might auto-activate it. The latest study partly supports this finding [13], yet a different one demonstrates that GPR61 is not a constitutively active receptor [14]. Another study has been conducted that described that some brain-expressed GPCRs, including GPR61, may be targets for plasmalogens [15].

Since the intrinsic activity of GPR61 is debatable and no further proofs have been shown that would confirm that 5-NOT is indeed an inverse agonist, in our article, we aimed to investigate 5-NOT-dependent and potential intrinsic activity of GPR61 in cAMP accumulation assay. This study was accompanied by in silico study on a GPR61-binding site.

Experimental

Cell Culture and Cloning

HEK293 stably expressing His-myc-GPR61 and control cells have been generated and characterized previously [6]. The cells were maintained in complete Dulbecco's Modified Eagle's Medi-

um (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin (Sigma Aldrich, 100 units/mL penicillin and 0.1 mg/mL streptomycin).

SDS/PAGE and Western Blotting
Cells were lysed in 25 mmol/L Tris (pH = 7.4) and resuspended in an equal volume of 2× radioimmunoprecipitation assay buffer and appropriate volume of 2× urea sample buffer. No >30 μg of protein was loaded and run on 4–20% Tris/glycine gels. Proteins were transferred onto PVDF membranes, which were then blocked in 10% nonfat milk for 2 h at room temperature. Membranes were incubated in 1:3,000 anti-myc (Cell Signaling Technology) or 1: 3,000 anti-GAPDH (Abcam, Cambridge, UK) in 5% nonfat milk overnight at 4 °C. Membranes were incubated subsequently in anti-mouse or anti-rabbit HRP-linked secondary antibody (Cell Signaling Technology) in 5% nonfat milk in wash buffer at room temperature for 2 h. The membrane was subsequently exposed to film and developed. Alternatively, the membrane was developed using ChemiDoc MP system imager (Bio-Rad).

cAMP Accumulation Assay
cAMP accumulation in the cells was measured using AlphaScreen kit (Perkin Elmer) according to the manufacturer's instructions. Briefly, HEK293 cells stably overexpressing His-*myc*-tagged GPR61 were seeded at 10^5 cells/well in a flat-bottom 96-well plate and incubated overnight at 37 °C. On the next day, the medium was removed, and the cells were carefully washed twice in 200 μL of stimulation buffer. Cells were incubated in the stimulation buffer for 20 min at 37 °C. In the next step, drugs were added, and the cells were incubated for 20 min at 37 °C. Adenylyl cyclase activator forskolin (1.0 μmol/L, Sigma-Aldrich) was then added, and the cells were stimulated for 10 min at 37 °C. Subsequently, the supernatants were removed, and the cells were lysed for 20 min in 1× immunoassay buffer. Subsequently, 5.0 μL of cell lysate was transferred to appropriate wells of OptiPlate 384-well plate (PerkinElmer). Acceptor beads were prepared at 1:25 dilution in the stimulation buffer and 5.0 μL of this beads suspension was added to lysate and left to incubate for 30 min in the dark at room temperature. In a separate tube mix of biotin-cAMP 1:240 and streptavidin-donor beads 1:150 was prepared and left to incubate for 30 min in the dark. After 30 min, 15 μL of biotin-cAMP and streptavidin-donor beads suspension was added. The plate was left for 4 h in the dark at room temperature before being read on PHERAstar FS HTS microplate reader (BMG LABTECH) using AlphaScreen module.

Homology Modeling
The homology model of inactive GPR61 was generated using the high-resolution structure of dopamine D4 receptor as a template (PDB ID: 5WIU, structural identity ~28%) [16]. The sequence of GPR61 (UniProt ID: Q9BZJ8) was aligned with that of D4R (UniProt ID: P21917) with ClustalX2 [17]. The N- and C-termini were excluded, and the alignment was manually edited to ensure the proper alignment of transmembrane domains. One hundred homology models of the GPR61 receptor were generated with MODELLER 9.19 [18], and the representative one was selected based on DOPE score.

Molecular Docking
Docking of 5-NOT was performed in AutoDock Vina [19] using default settings except for num_modes (20) and exhaustiveness (100). Protein and ligands protonation states were set for pH 7.4 with PROPKA [20] and Avogadro [21], respectively. The size of the binding box was set to 30 × 30 × 30 $Å^3$. The docking results were analyzed using LigPlot + [22] and PyMOL (The PyMOL Molecular Graphics System, version 2.0 Schrödinger, LLC).

Statistics and Data Analysis
The statistical analysis and curve fitting were done using Prism 6 (GraphPad). Throughout the manuscript, data are expressed as a mean ± SEM of *n* independent experiments (biological replicates). The Mann-Whitney test was used to test for significant differences between 2 groups, and Kruskal-Wallis test with Dunn's post hoc analysis was used to test for significant differences between >2 groups when $n \geq 3$, $^{**, \#\#}$ $p < 0.01$, $^{*, \#}$ $p < 0.05$.

Results and Discussion

In the present study, we use the HEK293 cells stably expressing His-*myc*-GPR61 (Fig. 1b) that have been previously characterized by us [6]. The data presented in Figure 2a reveal that the control HEK293 cells and HEK293 cells stably expressing the GPR61 receptor (HEK-GPR61) display similar basal cAMP levels. Differences in cAMP levels between the 2 cell lines, however, were only evident following stimulation with forskolin (Fig. 2a) arguing for receptor's synergistic action with gαs subunit.

The potential of 5-NOT (Fig. 2b) to act as a low-affinity GPR61 ligand was confirmed. Thus, 5-NOT significantly inhibited the forskolin-stimulated cAMP synthesis within HEK-GPR61 cells (Fig. 2c), consistent with previous pharmacological studies and predictions where 5-NOT acts as an inverse agonist of GPR61 and that GPR61 couples to $G\alpha_s$ as a cognate G protein. What is more, 5-NOT did not reduce forskolin-induced cAMP levels in the control cells, arguing for the specificity of this compound as a GPR61 ligand (Fig. 2d). Forskolin used at a lower concentration of 1 μmol/L did not elicit measurable differences in cAMP levels in control HEK cells, therefore, it was used at 10 μmol/L, concentration which is consistent with previously published reports on similar applications [23]. In our assay, 5-NOT did not significantly reduce forskolin-induced cAMP in the control cells. The application of forskolin at higher concentrations (e.g., 100 μmol/L) was not possible because of a high final DMSO concentration (1%).

In our model, as mentioned before, we have not been able to confirm the higher basal activity of the receptor, as opposed to the most recent study that has reported such a phenomenon [13]. However, our data, to some extent, support the results of the older paper [14]. The reasons for those differences are debatable, but in both cited

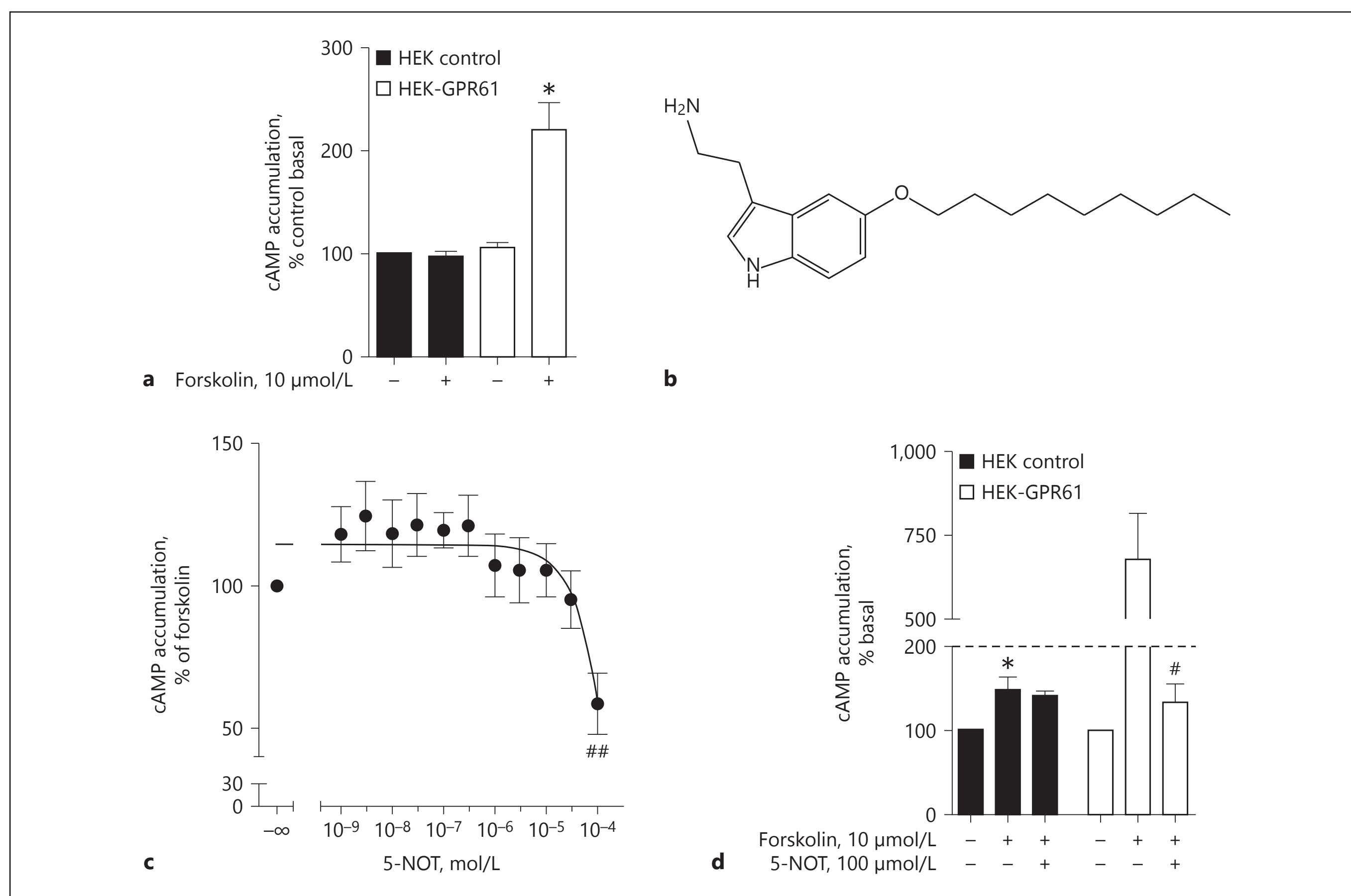

Fig. 2. a cAMP accumulation in HEK293 cells. Forskolin (1 μmol/L) induces a significant increase in cAMP production in the cells stably overexpressing GPR61, *n* = 4. **b** Structure of 5-NOT; a putative ligand of GPR61. **c** 5-NOT is a low-affinity inverse agonist of GPR61, *n* = 6. **d** Higher concentration of forskolin (10 μmol/L) is required to induce cAMP production in the control cells. 5-NOT (100 μmol/L) does not have an impact on cAMP levels in these cells; HEK-GPR61 cells were used for comparison to demonstrate a visible difference in response to 10 μmol/L forskolin, *n* = 3; * differences between forskolin- and vehicle-treated samples, # differences between forskolin only and forskolin + 5-NOT-treated samples.

papers, different assays were performed, and transiently transfected cells were used to measure the intrinsic activity of GPR61. It has to be noted that receptor levels have great impact on its basal activity and ligand-induced signaling. What is more, it is well-known that stably transfected cell system will behave differently to a transiently transfected one, especially in the signaling readouts. Lastly, we cannot exclude the GPR61 heterodimerization effect on the presented ligand-bound GPR61 mediated cellular responses [6].

We have also attempted to evaluate the binding of 5-NOT to GPR61 by molecular docking (GPR61 model was based on the dopamine receptor D4 structure, Fig. 3a). To analyze the docking results, we used the lowest-energy binding pose of 5-NOT in the predicted receptor binding pocket (AutoDock Vina calculated energy: –8.0 kcal/moL, Kd = 1.35 μmol/L; Fig. 3b). The long nonpolar tail of 5-NOT can directly interact with side chains of amino acids of TM5 via hydrophobic interactions. Also, the pose of 5-NOT in the binding pocket supports hydrogen bond formation with neighboring $L93^{2.57}$, C195 in ECL2 and $Y303^{6.51}$ (Fig. 3b).

Conclusion

In conclusion, the results presented here reveal that GPR61 does not have an increased basal activity, but its overexpression promotes a higher cellular response – cAMP accumulation – to forskolin. We also confirm that

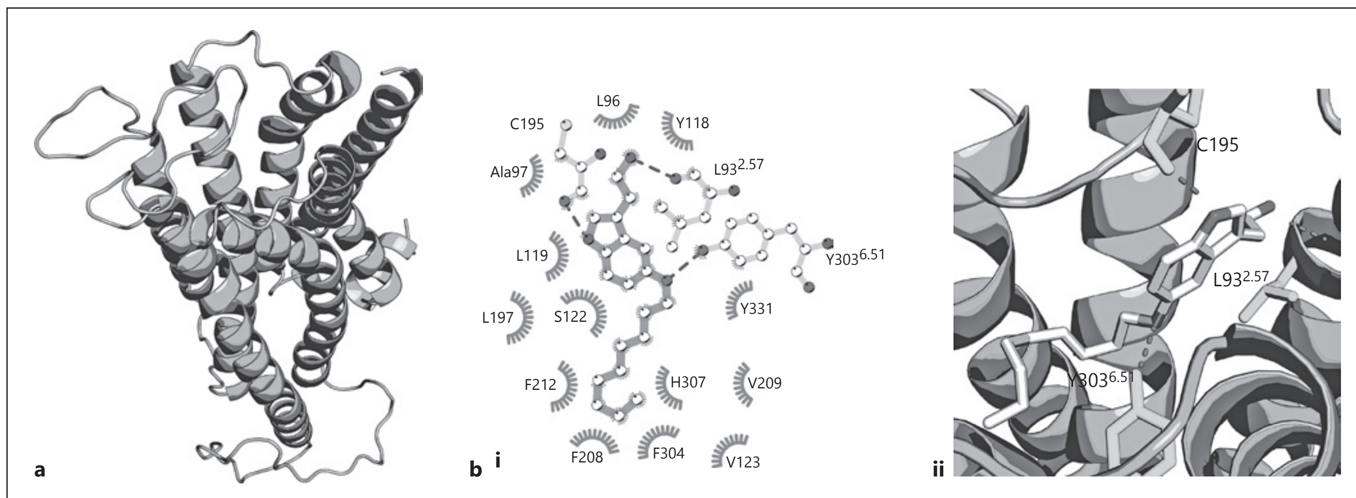

Fig. 3. a GPR61 receptor model. Binding poses of 5-NOT (**b**) with all the interacting neighboring residues (**i**) and hydrogen bond interacting residues (**ii**).

5-NOT is a low-affinity inverse agonist. Additionally, we demonstrate that 5-NOT could bind in the intra-TM orthosteric binding site of GPR61. We have previously shown that GPR61 is highly expressed in Th17 cells, and given that, under certain conditions, it can have a high intrinsic activity – the development of inverse agonist targeting this GPCR is desirable [6]. We consider that our findings may contribute to the process of GPR61 deorphanization.

Disclosure Statement

The authors declare that they have no conflict of interest.

Author Contributions

P.K., G.G., and N.M.B. conceived the project and designed the experiments. P.K. performed all the experiments. P.K. and J.J.S.-K. analyzed the data. P.K. and N.M.B. wrote the paper. N.M.B. supervised the study.

References

1 Hauser AS, Chavali S, Masuho I, Jahn LJ, Martemyanov KA, Gloriam DE, et al. Pharmacogenomics of GPCR Drug Targets. Cell. 2018 Jan;172(1–2):41–54.e19.

2 Pándy-Szekeres G, Munk C, Tsonkov TM, Mordalski S, Harpsøe K, Hauser AS, et al. GPCRdb in 2018: adding GPCR structure models and ligands. Nucleic Acids Res. 2018 Jan; 46 D1:D440–6.

3 Cikos S, Gregor P, Koppel J. Cloning of a novel biogenic amine receptor-like G protein-coupled receptor expressed in human brain. Biochim Biophys Acta. 2001 Oct;1521(1–3): 66–72.

4 Lee DK, George SR, Cheng R, Nguyen T, Liu Y, Brown M, et al. Identification of four novel human G protein-coupled receptors expressed in the brain. Brain Res Mol Brain Res. 2001 Jan;86(1–2):13–22.

5 Haris Shaikh L, Zhou J, Azizan EAB, Kuc RE, Davenport AP, Brown MJ. Investigation of zonal regulation of steroidogenisis in the human adrenal with emphasis on GPR61, a potential candidate regulator of aldosterone secretion. BPS Winter Meeting 2012, 133P Queen Elizabeth II Conference Centre London; 2012.

6 Kozielewicz P, Alomar H, Yusof S, Grafton G, Cooper AJ, Curnow SJ, et al. N-glycosylation and expression in human tissues of the orphan GPR61 receptor. FEBS Open Bio. 2017 Nov;7(12):1982–93.

7 Nambu H, Fukushima M, Hikichi H, Inoue T, Nagano N, Tahara Y, et al. Characterization of metabolic phenotypes of mice lacking GPR61, an orphan G-protein coupled receptor. Life Sci. 2011 Nov;89(21–22):765–72.

8 Trzaskowski B, Latek D, Yuan S, Ghoshdastider U, Debinski A, Filipek S. Action of molecular switches in GPCRs–theoretical and experimental studies. Curr Med Chem. 2012; 19(8):1090–109.

9 Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. Methods Neurosciences. 1995;25: 366–428.

10 Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U, et al. Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. J Biol Chem. 2001 Aug;276(31): 29171–7.

11 Takeda S, Yamamoto A, Okada T, Matsumura E, Nose E, Kogure K, et al. Identification of surrogate ligands for orphan G protein-coupled receptors. Life Sci. 2003 Dec;74(2–3): 367–77.

12 Toyooka M, Tujii T, Takeda S. The N-terminal domain of GPR61, an orphan G-protein-coupled receptor, is essential for its constitutive activity. J Neurosci Res. 2009 May;87(6): 1329–33.

13 Oishi A, Karamitri A, Gerbier R, Lahuna O, Ahmad R, Jockers R. Orphan GPR61, GPR62 and GPR135 receptors and the melatonin MT2 receptor reciprocally modulate their signaling functions. Sci Rep. 2017 Aug;7(1): 8990.

14 Martin AL, Steurer MA, Aronstam RS. Constitutive Activity among Orphan Class-A G Protein Coupled Receptors. PLoS One. 2015 Sep;10(9):e0138463.

15 Hossain MS, Mineno K, Katafuchi T. Neuronal Orphan G-Protein Coupled Receptor Proteins Mediate Plasmalogens-Induced Activation of ERK and Akt Signaling. PLoS One. 2016 Mar;11(3):e0150846.

16 Wang S, Wacker D, Levit A, Che T, Betz RM, McCorvy JD, et al. D4 dopamine receptor high-resolution structures enable the discovery of selective agonists. Science. 2017 Oct;358(6361):381–6.

17 Higgins DG, Thompson JD, Gibson TJ. Using CLUSTAL for multiple sequence alignments. Methods Enzymol. 1996;266:383–402.

18 Sali A, Potterton L, Yuan F, van Vlijmen H, Karplus M. Evaluation of comparative protein modeling by MODELLER. Proteins. 1995 Nov;23(3):318–26.

19 Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 2010 Jan;31(2):455–61.

20 Olsson MH, Søndergaard CR, Rostkowski M, Jensen JH. PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pKa Predictions. J Chem Theory Comput. 2011 Feb;7(2):525–37.

21 Hanwell MD, Curtis DE, Lonie DC, Vandermeersch T, Zurek E, Hutchison GR. Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. J Cheminform. 2012 Aug;4(1):17.

22 Laskowski RA, Swindells MB. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. J Chem Inf Model. 2011 Oct;51(10):2778–86.

23 Beazely MA, Alan JK, Watts VJ. Protein kinase C and epidermal growth factor stimulation of Raf1 potentiates adenylyl cyclase type 6 activation in intact cells. Mol Pharmacol. 2005 Jan;67(1):250–9.