A simple open source bioinformatic methodology for initial exploration of GPCR ligands’ agonistic/antagonistic properties

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Abstract
Drug development is an arduous procedure, necessitating testing the interaction of a large number of potential candidates with potential interacting (macro)molecules. Therefore, any method which could provide an initial screening of potential candidate drugs might be of interest for the acceleration of the procedure, by highlighting interesting compounds, prior to in vitro and in vivo validation. In this line, we present a method which may identify potential hits, with agonistic and/or antagonistic properties on GPCR receptors, integrating the knowledge on signaling events triggered by receptor activation (GPCRs binding to Gα, β, γ proteins, and activating Gαq exchanging GDP for GTP, leading to a decreased affinity of the Gα for the GPCR). We show that, by integrating GPCR-ligand and Gα-GDP or -GTP binding in docking simulation, which correctly predicts crystallographic data, we can discriminate agonists, partial agonists, and antagonists, through a linear function, based on the ∆G (Gibbs-free energy) of liganded-GPCR/Gα-GDP. We built our model using two Gαq (α2-adrenergic and prostaglandin-D2), four Gαi (μ-opioid, dopamine-D3, adenosine-A1, rhodopsin), and one Gαo (serotonin) receptors and validated it with a series of ligands on a recently deorphanized Gαi receptor (OXER1). This approach could be a valuable tool for initial in silico validation and design of GPRC-interacting ligands.

KEYWORDS
agonist, antagonist, biological activity prediction, docking, GPCR, in silico, OXER1

1 | INTRODUCTION

Progress in biochemistry and cell biology resulted in a better understanding of the events and necessary steps involved in the interaction of a cell with an administered drug substance, leading to the discovery and/or synthesis of novel pharmaceuticals. However, a novel drug development continues to be slow (FDA approved 59 novel drugs in 2018,1 including biological factors), as it involves the

Abbreviations: 5-oxo-ETE, 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid; cAMP, Cyclic adenosine monophosphate; GDP, Guanosine diphosphate; GPCR(L), Ligand-bound GPCR; GPCR, G-protein-coupled receptor; GTP, Guanosine-5'-triphosphate; Gα, G alpha subunit; OXER1, Oxo-eicosanoid Receptor 1; pdb, protein data bank; pdbqt, Protein Data Bank, Partial Charge (Q), & Atom Type (T); RMSD, Root mean square deviation; ∆G, Difference in Gibbs-free energy.

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testing of an increasing number of chemical libraries for positive hits and the subsequent biological validation of promising candidates. Therefore, any progress leading to an initial discrimination of novel potential bioactive compounds could lead to a more accurate identification of possible novel drug candidates.

Of the 59 drugs approved in 2018, 23 target membrane components/receptors, whereas 11 target G-protein-coupled receptors (GPCR). Membrane receptors act as conveyors of extracellular signals into the cell. Membrane receptors can be distinguished as one-pass single-chain proteins, acting as mono-oligomers and multiple passes proteins. Among the latter, the seven transmembrane helix (7TM) GPCR family contains ~800 members overall (of which ~400 are olfactory receptors). GPCRs are involved in different signal transduction pathways, triggered by a plurality of extracellular signals (including photons, light-sensitive compounds, photons, odorants, pheromones, hormones, neurotransmitters, and a number of ligands, varying in size from small molecules to peptides to large (glycol) proteins). GPCR-initiated signal transduction results in many physiological processes, interfering with the (path)physiology of many systems, such as the endocrine (including the reproductive), neurological or cardiovascular systems. Such a wide impact, makes GPCR a preferential drug target candidate group. Indeed, GPCR-interacting drugs account for ~34% of the global market share. However, only a small fraction of GPCRs (206 entries according to https://gpcrdb.org/structure/statistics) have been crystallized to date, making difficult the prediction of novel pharmacological substances.

Molecular docking plays a major role in identifying molecules that might fulfill the requirements of drug development. The applied methodologies simulate the interaction of ligands (small molecules or peptides) with corresponding receptors, in monomeric or oligomeric states. The derived solutions are represented as scoring function (usually reported as the difference in Gibbs-free energy for receptor preparation, (B) ligand-receptor docking and (C) G protein interaction (Figure 1 and Supporting Information 2, which provides an illustrated User’s Manual).

2 | MATERIAL AND METHODS

2.1 | In silico methods

Our approach consists of three sequential phases: (A) Ligand and receptor preparation, (B) ligand-receptor docking and (C) G protein interaction (Figure 1 and Supporting Information 2, which provides an illustrated User’s Manual).

2.1.1 | Ligand and Receptor Preparation

- For receptor preparation: the sequence of human receptors, in fasta format, were retrieved from the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/) and introduced to the Swiss Model Biospace (http://swissmodel.expasy.org/interactive).
- If a crystalized receptor file (bound or not with an agonist or an antagonist) was available, the system returned the code (and corresponding pdb 3D coordinates file of the receptor or its complex with an agonist or an antagonist, from data stored in the Protein Data Bank (https://www.rcsb.org/)). When the structure contained a ligand, the receptor structure was manually extracted (using a text editor) from the returned pdb file. This did not interfere with the subsequent (flexible) binding, as a full backbone and side chain flexible binding was performed (see below). All protein pdb codes, used in this study, are presented in Table S1.
- If a crystalized structure was not available, the 3D structure of the receptor was simulated by molecular modeling calculations. In this case, the fasta receptor file was introduced in the Swiss Model Biospace (http://swissmodel.expasy.org/interactive), which returned a series of files/crystalized solutions with a variable coverage homology based on the sequence identity to the file in question. We have retained solutions with a coverage homology ≥ 50%-70%. Please refer to the Swiss Model Biospace for further details of the modeling methodology.
- Known ligands were retrieved from the ZINC database (http://zinc.docking.org/), usually in a canonical smiles format. Novel molecules were designed in ChemBioDraw (v12.0, Perkin Elmer, Boston, MA, free for Academic use from the University of Cambridge, any other chemical drawing program, such as ChemSketch (https://www.acdlabs.com/resources/freeware/chemsketch/), BKChem (http://bkchem.zirael.org/) or Symyx Draw (https://symyx-draw.jaleco.com/) can be used at this stage) and the structures were also translated in canonical smiles format. Subsequently, pdb files were created with the Open Babel program (http://openbabel.org/).

Ligands (agonists, partial agonists,
and antagonists) for each receptor were retrieved from the gene cards web resource (www.GeneCards.org).\textsuperscript{19}

2.1.2 Ligand-receptor docking experiment

Flexible docking algorithms can be broadly divided into methods, in which flexibility is attained during the ligand-binding interaction (on-the-fly methods) and methods applying multiple receptor or ensemble poses, at the beginning or during the simulations (see,\textsuperscript{6} for a discussion). As our goal was to provide a solution, applicable to known or novel GPCRs, in which experimental and/or crystallographic data might not be available, we have opted for on-the-fly approach. We have used the online server Galaxy7TM (galaxy.seoklab.org), in which a full on-the-fly ligand and receptor flexibility is implemented.\textsuperscript{20} First, we have used the server for the prediction of the possible binding grooves of each molecule. In our approach, we have restricted our results to only the orthosteric binding site of the molecule (module GalaxySite). Subsequently, we performed a fully flexible binding of the ligand and receptor molecules (module Galaxy7TM). The server uses an algorithm, based on the GalaxyDock2 docking,\textsuperscript{21} which, after an automatic prediction of the ligand binding pocket, permits a full ligand/receptor flexibility during binding simulation. This step is followed by optimization and subsequent refinement, through a specific algorithm named GalaxyRefine,\textsuperscript{22,23} which permits a protein-ligand structure refinement, by applying iterative side chain re-packing and overall structure relaxation.\textsuperscript{20,22,23} returning the pdb files of the best ligand-receptor solutions. The 3D structures of the liganded and unliganded receptor were compared, using the UCSF Chimera 1.11.2 program,\textsuperscript{24} available from https://www.cgl.ucsf.edu/chimera/. In cases where a crystal was retrieved, the retained solution was compared with the crystal structure. In addition, special attention was paid to confirm that ligands were
bound to the orthosteric binding pocket of the corresponding GPCR. Finally, we have compared the correct pose of the ligands, by extracting them from the crystal structure and the retained solution, with a text editor, and compared their RSMD with the Chimera program.

2.1.3 | G-protein interactions

A subsequent step of GPCR-ligand activation is the binding with G-proteins, specific sites. More specifically, \( G_\alpha \) is bound to intracellular activated receptor loops 2 and 3, and \( G_{\beta\gamma} \) is bound to intracellular chain 8,25,26 initiating specific intracellular signaling events. In this work, we have examined the interaction of GPCRs with \( G_\alpha \) proteins. It is to note that, after binding of \( G_\alpha \) protein (bound to GDP and denoted here as \( G_\alpha\*-GDP \)), the nucleotide is exchanged, after receptor activation, to GTP (\( G_\alpha\*-GTP \)), and the \( G_{\beta\gamma} \)-GTP protein is liberated (due to a decrease of affinity for the GPCR) and subsequently triggers specific signaling pathways. Here, we simulated the interaction of known and novel GPCRs with \( G_\alpha \) proteins.

At a first step, we have retrieved the sequences of \( G_\alpha \)-proteins, in fasta format, from the NCBI protein database, and after a SwissDock generation of a 3D structure, with known crystal templates, were introduced to the GalaxyWEB to generate and refine the structures, as discussed above for the receptor files. This step was necessary, as the reported crystal structures of the different \( G_\alpha \) molecules may contain significant gaps. The retrieved structures of \( G_\alpha \)-proteins were then docked with GDP or GTP in a fully flexible on-the-fly method, in the GalaxyWEB server and the liganded \( G_\alpha \) pdb files were recovered. The same controls, as for the ligand-GPCR binding were performed for the \( G_\alpha\*-GDP \) or GTP retained solutions (comparison of the structures by superposition, ligand pose comparison).

The ligand-receptor and \( G_\alpha\*-GDP \) or \( G_\alpha\*-GTP \) pdb files were used as input in the Hex 8.0.8 program (http://hex.loria.fr/),27 a specialized, locally executed, program, for protein-protein, or protein-nucleic acid interactions, based on a spherical rotated protein complexes, taking into account both surface shape and electrostatic charge. Hex returns, through a graphical user interface, a set of > 100 solutions, with the corresponding \( \Delta G \) values. We have manually inspected and retained only solutions (usually scored first) in which \( G_\alpha \) molecules bind to GPCRs intracellular loops 2 and 3.

2.2 | Validation of the obtained solutions

The obtained GPCR-\( G_\alpha \) models, obtained from the above procedure, were compared with the reported structures of liganded receptor-\( G_\alpha \) proteins. We have retrieved data for the liganded \( \beta \)-adrenergic receptor (PDB code 3SN6),28 the \( \mu \)-opioid receptor (PDB code 6DDE),29 the rhodopsin receptor (PDB code 6CMO),30 the serotonin receptor (PDB code 6G79),31 the adenosine A1 receptor (PDB code 6D9H),32 co-crystallized with corresponding Go proteins. Data were inspected in UCSF Chimera program, by superpositioning of the two structures and the corresponding total and local RMSD value (in Å) were retrieved, with Needleman-Wunsch alignment33 and with the use of BLOSUM-62 matrix.33

2.3 | In vitro validation assay

As our goal was to use the proposed algorithm as a prediction tool for the agonistic or antagonistic character of novel ligands on specific GPCRs, we have further validated our in silico results, by exploring the interaction of a series of pregnenolone analogs34 and polyphenol molecules35, as agonists or antagonists of the novel orphanized GPCR OXER1.36,37 OXER1 is an oxo-eicosanoid receptor, on which 5-oxo-ETE is reported to be the physiological agonist and which can also bind other oxo-eicosanoids, products of arachidonic acid cellular transformation. Recently, we have identified this receptor as a membrane androgen binding site,38 with testosterone acting antagonistically on cAMP production and kinases signaling. It is to note that OXER1 binds to a \( G_\alpha \) protein and decreases intracellular cAMP production, whereas testosterone, in equimolar concentration, reverts this inhibition by ~50%.38 Therefore, in order to validate our in silico data, we have assayed cAMP production in DU145 human prostate cancer cells, bearing OXER1, according to our previous report.38

Cells (from Braunschweig, Germany) were cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS), at 37°C, 5% CO2. All media were purchased from Invitrogen (Carlsbad, USA) and all chemicals from Sigma (St. Louis, MO), unless otherwise stated. 5-oxo-ETE (5-Oxo-(6E,8Z,11Z,14Z)-6,8,11,14-eicosatetraenoic acid), was from Tocris, Testosterone (Sigma Aldrich), TC150, TC151, and TC153 were synthesized at the Institute of Chemical Biology, National Hellenic Research Foundation, Athens, Greece, B2 and B5 polyphenols were obtained from Professor J. Vercauteren (University of Montpellier, France), whereas Epicatechin was purchased from Sigma Aldrich.

The cyclic adenosine monophosphate (cAMP) production after OXER1 stimulation by 5-oxo-ETE (10−7M) alone, or in the presence of testosterone or the other compounds (10−4M, see Results) was examined, with a gain-of-signal competitive immunoassay (Promega cAMP Glo TM, Madison, WI). Since OXER1 is a \( G_\alpha \)i coupled receptor, forskolin (15 \( \mu \)M) was used to stimulate cAMP production and reveal the inhibitory effect of 5-oxo-ETE. The antagonistic effect of testosterone and other agents was assayed as follows (Figure S3): cells were pretreated with the different compounds at a concentration of 10−4M for 15 min at 37°C, prior to the addition of 5-oxo-ETE, and cAMP was further assayed. The produced luminescence signal was read in a Microplate Fluorescence Reader (BIO-TEK Instruments Inc Winowski, Vermont, USA). Results were expressed as % reversion of the 5-oxo-ETE effect, in the presence of forskolin.38
2.4 | Statistical Analysis

Discriminant analysis was performed with the SPSS V21 program (IBM, SPSS Statistics), whereas group comparisons were made with the GraphPad Prism V6.0.5 (GraphPad Software Inc). A statistical threshold of \( P < .05 \) was retained for significance.

3 | RESULTS

3.1 | Implementation of the proposed bioinformatic solution

3.1.1 | Training set

Ligand-Receptor interaction

At a first step, we have performed an in silico docking of known small molecules (total number: 78), on six different human GPCRs, crystalized or not, as our method was oriented towards the identification of novel substances for human diseases. We have used the crystalized \( \beta_2 \)-adrenergic (pdb 3SN6 \(^{28} \)), dopamine \( D_1 \) (pdb 3PBL \(^{39} \)), \( \mu \)-opioid (pdb 6DDE \(^{29} \)), adenosine \( A_3 \) (pdb 6D9H \(^{32} \)), and rhodopsin (pdb 6CMO \(^{30} \)) receptors. For prostaglandin \( DR_2 \) receptors, as a crystal was not available, our model was based on the human \( C_{5a} \) anaphylatoxin chemotactic receptor 1, pdb 5O9H \(^{40} \) (see Material and Methods for details). The receptor and ligand molecules (in pdb format) were then introduced in the Galaxy 7TM server, and a fully flexible (ligand and receptor) binding was performed.

Results (as changes of the Gibbs-free energy changes, \( \Delta G \), in kcal/mol) are shown in Tables 1-3, column GPCR-Ligand.

Interaction of the ligand-receptor complex with \( G_{a} \)-proteins

A subsequent step following ligand-GPCR interaction is the binding of the liganded receptor to the heteroprotein complex \( G_{a}\rightarrow \alpha_\text{i-} \) \(^{11-13} \) triggering specific signaling events. \( G_{a} \) proteins interact with intracellular loops 2 and 3, whereas \( G_{b\gamma} \) with intracellular loop 3 and the intracellular C-terminal helix. \(^{8} \) \( G_{a} \) proteins are bound to guanine nucleotides; specifically, \( G_{a} \) proteins are bound to GDP, and in this form, they interact with the ligand-activated GPCR (GPCR(L)). The affinity of the GPCR(L)-\( G_{a} \) decreases substantially when an exchange of GDP by GTP occurs, leading to \( G_{a} \) dissociation from the GPCR(L) complex and the initiation of intracellular signaling events. \(^{25} \)

Interaction of the liganded receptor with \( G_{a} \)-GDP and \( G_{a} \)-GTP complexes is also shown in Tables 1-3. We have used \( G_{a}\rightarrow \rightarrow \), \( G_{a}\rightarrow \), or \( G_{a}\rightarrow \) molecules according to the reported physiological interaction of each receptor with \( G_{a} \) proteins. In this case, as the two interacting structures are macromolecules, the resulting \( \Delta G \) values were much higher. \(^{21} \) When GDP was exchanged for GTP on

| RECEPTOR | LIGAND | GPCR-Ligand (kcal/mol) | GPCR(L)-\( G_{a} \)-GDP (kcal/mol) | GPCR(L)-\( G_{a} \)-GTP (kcal/mol) | EFFECT
|-----------|--------|------------------------|-----------------------------------|-----------------------------------|--------
| \( \beta_2 \)-adrenergic | Bitotolterol | –16.7 | –910.6 | –700.1 | Ago/Ago
| | Formoterol | –13.4 | –877.5 | –700.9 | Ago/Ago
| | Isoprenaline | –10.8 | –933 | –699 | Ago/Ago
| | Levosalbutamol | –12 | –890.7 | –695 | Ago/Ago
| | Orciprenaline | –11.1 | –892.3 | –696 | Ago/Ago
| | Ritodrine | –11.8 | –924.4 | –704.4 | Ago/Ago
| | Salbutamol | –11.4 | –961.1 | –691.3 | Ago/Ago
| | Salmeterol | –16.8 | –956.9 | –695.2 | Ago/Ago
| | Terbutaline | –11.2 | –853.1 | –697 | Ago/Ago
| | ICI118,551 | –10.6 | –725.1 | –707.5 | Antago/PA
| | Butoxamine | –12.1 | –706.4 | –696.6 | Antago/PA
| | Propranolol | –11.6 | –719.7 | –706.6 | Antago/PA
| | BI-167107 | –15.4 | –971.3 | –705.6 | Ago/Ago
| Prostaglandin \( DR_2 \) | Prostaglandin-\( E_2 \) | –14.3 | –1079 | –475.6 | Ago/Ago
| | Prostaglandin-\( F_{2a} \) | –14.7 | –1091.9 | –431.3 | Ago/Ago
| | Prostacyclin | –14.6 | –1003.2 | –455 | Ago/Ago
| | Fevipiprant | –14.9 | –462.2 | –412.2 | Antago/Antago
| | Ramatroban | –15.4 | –422.1 | –416.4 | Antago/Antago
| | Setipiprant | –14.9 | –442.8 | –418 | Antago/Antago

Abbreviations: Ago, Agonist; Antago, Antagonist; GPCR(L), Ligand-bound GPCR; PA, Partial Agonist.
TABLE 2  Fully flexible ligand binding results on dopamine D₃ (pdb 3PBL), μ-opioid (pdb SC1M), Adenosine A₁ (pdb 6D9H), and Rhodopsin (pdb 6CMO) receptors, together with the liganded GPCR (GPCR(L)) binding to Gᵢ in its GDP and GTP-bound forms. All data are reported as differences in the Gibbs-free energy (ΔG), expressed in kcal/mol. The effect column presents the reported action of the compound (bibliography) and the predicted effect by the proposed model (Model). See text for details.

| RECEPTOR | LIGAND               | GPCR-Ligand (kcal/mol) | GPCR(L)-Gₐ GDP (kcal/mol) | GPCR(L)-Gₐ GTP (kcal/mol) | EFFECT   | Bibliography/Model |
|----------|-----------------------|------------------------|---------------------------|---------------------------|----------|--------------------|
| Dopamine D₃ | Dopamine              | -9.6                   | -779.9                    | -349                      | Ago/PA   |                    |
|          | Quinpirole            | -9.8                   | -719.7                    | -378.8                    | Ago/PA   |                    |
|          | SOH-DPAT              | -8.8                   | -864                      | -348.4                    | Ago/Ago  |                    |
|          | Pergolide             | -9.4                   | -780.2                    | -400.7                    | Ago/PA   |                    |
|          | Captodiamine          | -10                    | -793                      | -311                      | Ago/PA   |                    |
|          | Apomorphine           | -6.7                   | -804.9                    | -376.3                    | Ago/Ago  |                    |
|          | Aripiprazole          | -16.9                  | -761.1                    | -327.7                    | PA/PA    |                    |
|          | Cariprazine           | -11.3                  | -763.6                    | -388.8                    | PA/PA    |                    |
|          | Buspirone             | -11.1                  | -772.3                    | -334.5                    | PA/PA    |                    |
|          | Paraclopride          | -5.2                   | -787.1                    | -349.4                    | PA/PA    |                    |
|          | Nafadotride           | -8.5                   | -652.5                    | -373.3                    | Antago/Antago |                |
|          | Raclopride            | -10.7                  | -685.4                    | -330                      | Antago/Antago |                |
|          | Haloperidol           | -10.5                  | -683                      | -321.6                    | Antago/Antago |                |
|          | Amisulpride           | -10.2                  | -686                      | -396.5                    | Antago/Antago |                |
|          | Cyproheptadine        | -6.1                   | -690.8                    | -285.7                    | Antago/Antago |                |
|          | Risperidone           | -12.1                  | -682.7                    | -370.7                    | Antago/Antago |                |
|          | Acetylumorphine       | -9.6                   | -855.2                    | -615.4                    | Ago/Ago  |                    |
|          | Benzhydrocodone       | -10.9                  | -821.3                    | -616.9                    | Ago/Ago  |                    |
| μ-opioid | Heroin                | -12.4                  | -1045.4                   | -620.7                    | Ago/Ago  |                    |
|          | Methadone             | -8.7                   | -902.4                    | -561.6                    | Ago/Ago  |                    |
|          | Nicocodeine           | -12.0                  | -855.1                    | -526.4                    | Ago/Ago  |                    |
|          | Butorphanol           | -10.2                  | -769.8                    | -540.4                    | PA/PA    |                    |
|          | Ciprefadol            | -9.0                   | -773.2                    | -656.4                    | PA/PA    |                    |
|          | Cyclorphan            | -8.9                   | -779.8                    | -669.2                    | PA/PA    |                    |
|          | Ketorfanol            | -9.0                   | -778.6                    | -638.6                    | PA/PA    |                    |
|          | Xorphanol             | -9.0                   | -783.3                    | -674.4                    | PA/PA    |                    |
|          | Moxazocine            | -8.5                   | -799.0                    | -629.7                    | PA/PA    |                    |
|          | Nalbuphine            | -10.1                  | -780.7                    | -589.9                    | PA/PA    |                    |
|          | Nalmefene             | -9.8                   | -697.2                    | -650.7                    | Antago/Antago |                |
|          | Naldeine              | -9.7                   | -703.1                    | -594.9                    | Antago/Antago |                |
|          | Nalorphine            | -10.7                  | -699.8                    | -651.2                    | Antago/Antago |                |
|          | Naloxone              | -9.3                   | -698.4                    | -575.7                    | Antago/Antago |                |
|          | Naltrexone            | -9.9                   | -691.3                    | -619.2                    | Antago/Antago |                |
|          | Levallorphan          | -8.8                   | -698.4                    | -584.0                    | Antago/Antago |                |
|          | DAMGO                 | -16.3                  | -861.0                    | -582.0                    | Ago/Ago  |                    |
| Adenosine A₁ | ADO               | -8.7                   | -1079.6                   | -646.1                    | Ago/Ago  |                    |
|          | CCPA                  | -12.5                  | -1062.6                   | -659.9                    | Ago/Ago  |                    |
|          | CPA                   | -11.9                  | -1009.9                   | -563.6                    | Ago/Ago  |                    |
|          | N(6)-Cyclohexyladenosine | -11.4                | -1099.7                   | -679.7                    | Ago/Ago  |                    |
|          | Tecadenoson           | -11.9                  | -888.4                    | -585.8                    | Ago/Ago  |                    |
|          | Selodenoson           | -13                    | -990.8                    | -638.6                    | Ago/Ago  |                    |
|          | Caffeine              | -6.3                   | -631.4                    | -372.6                    | Antago/Antago |                |

(Continues)
the $G_a$ proteins (Tables 1-3), the interaction of $G_a$ proteins with the receptor was significantly decreased. In this case, the obtained values do not differ among agonists, partial agonists, and antagonists (Figure 2).

Verification of the GPCR-ligand binding and $G_a$ interaction

In view of the potential application of the proposed tool for the identification of possible small molecule agonistic and antagonistic candidates for GPCRs, we have performed a number of verifications of our approach:

First, we simulated the interaction of unliganded GPCRs with either nonliganded $G_{as/o}$ proteins or $G_{as/o}$ proteins bound to either GDP or GTP. The same test was performed with liganded GPCRs (Table S2). We show that unliganded GPCRs interact with a substantially lower affinity, or do not interact at all, with either nonliganded or GDP/GTP-bound $G_a$ proteins. In addition, we show that liganded GPCRs do not interact with nonliganded $G_{as/o}$ proteins. This result confirms the validity of our approach, which is in line with the physiological function concerning the initiation of signaling by GPCRs.11-13

Second, we have compared the retained solutions of the ligand receptor complexes with those deposited in the PDB database. In all cases, a very small RMSD was found between the simulated solution and the crystal structure (Specific examples are presented in Figure S1), suggesting the very good match of our simulation with crystallography-obtained data.

Third, we have compared (whenever possible) the simulated structure of the complex [GPCR-L]-[G$_a$-GDP] with available crystals (human $\beta$-adrenergic (pdb 3SN6),28 the $\mu$-opioid (pdb 6DDE),29 the rhodopsin (pdb 6CMO),30 the serotonin (pdb 6G79),31 and the adenosine A$_1$ receptor (pdb 6D9H),32 co-crystalized with the corresponding $G_a$ proteins) (Figure S2). We report that, a very close match for all solutions, with the exception of the rhodopsin receptor (RMSD 15.4 Å). In the latter, a very good match was found at the interacting part of the $G_a$ protein, whereas the observed differences in $G_a$ proteins might be attributed to the recalculation of these proteins’ 3D structure, due to major disruptions of the protein structures in the $G_a$ crystal.

Finally, we have tried to simulate $G_a$-GDP binding to an unrelated multipass membrane protein (aquaporin monomer, extracted
from pdb 6KXW). All three Gα-GDP complexes did not interact with this protein monomer, corroborating about the specificity of the simulated interaction.

In view of the above, we have concluded that our approach may indeed correctly simulate the interaction of known GPCRs with their corresponding Gα-proteins.

**Gα binding to the liganded receptor can discriminate between GPCR agonists and antagonists**

After determining the different ΔGs for ligand-GPCR binding and for liganded GPCR-liganded Gα interaction (presented in Tables 1-3), we explored whether these data could be used for the prediction of agonistic or antagonistic properties of the different ligands. A backward elimination discriminant analysis, with the 78 compounds presented here and their reported agonistic, antagonistic, or partial agonistic properties retained only ΔG of the liganded GPCR-GDP-bound Gα protein as a significant discriminant element. A linear function of this factor (0.010 x ΔG GPCR(L)-Gα_GDP + 7.895) was sufficient to correctly discriminate 93% of antagonists, 87% of partial agonists, and 88% of agonists (F = 84.089, P = 4.3125). Using group centroids, we have estimated the cut-offs of the three groups (agonists, partial agonists, and antagonists), through a weighted mean calculation (Weighted Mean n=((Mean1xN1) + (Mean2xN2))/(N1 + N2), where N is the number of substances used for the calculation). A cut-off of 1.231 (corresponding to a ΔG GPCR(L)-Gα_GDP of −666 kcal/mol) between antagonists and partial agonists and a cut-off of −0.978 (corresponding to a ΔG GPCR(L)-Gα_GDP of −887 kcal/mol) between partial agonists and full agonists was calculated. This prediction is reported in the last column of Tables 1-3. As shown, in the majority of cases (70/80, 87.5%) a correct classification was obtained; however, in 8/80 cases, reported action and prediction were not obtained. Inspection of the chemical structures of misclassified substances did not provide a valid clue about this misclassification. However, in the majority of cases, these misclassified compounds have a receptor-Gα value near the cut-off of the different categories. We presume that, with a better calculation of the classification intervals with a larger number of compounds and/or GPCRs, this 12.5% mis-classification might improve.

### 3.2 Validation set

#### 3.2.1 Classification of novel compounds

At a first step, we have retrieved, from the list of FDA-approved drugs for 2017 and 2018, four compounds, characterized as agonists or antagonists of GPCRs (Prucalopride as a selective 5HT4 receptor, Lofexidine as an agonist of α2A adrenergic receptor, Latanoprostene as a selective agonist of PgF receptor, and Naldemedine as a µ-opioid receptor antagonist). We have applied our method, in order to provide agonistic or antagonistic properties of the compounds (Table 4). We have verified whether these drugs could interact with the receptor we have analyzed in this work. Surprisingly, all compounds interact with other GPCR subtypes, with a relative high affinity, docked to the correct ligand binding pocket of each molecule. However, neither Prucalopride, nor Lofexidine or Latanoprostene induce a binding of the corresponding Gα protein. In contrast, Naldemedine induces a Gα binding, with an affinity of −657 kcal/mol, correctly classifying it as an opioid receptor antagonist.

At a second step, we have investigated the interaction of Prucalopride, Lofexidine, and Latanoprostene with 5HT4 receptor, α2A adrenergic and PgF receptor, respectively. Neither of these three human receptors have been crystallized yet. We have therefore used the Swiss Model Biospace to provide the most promising solution of its 3D structure for each receptor, by introducing each receptor sequence in fasta format. The best returned solutions were based on crystals 6AK3 (prostaglandin E receptor) for PgF receptor, crystal 5V54 (5-HT1B receptor) for the α2A-Adrenergic Receptor, and crystal 3PDS (B2 adrenoreceptor) for 5-HT4. These models have further been refined (and completed whenever necessary) in the Galaxy Refine routine of the Galaxy Web server, and binding of the corresponding compounds was performed, followed by the binding of the GDP- or GTP-bound corresponding Gα protein (the
Hence, we used our approach to predict the agonistic-antagonistic properties of both crystalized or not GPCRs. As shown in Table 5, docking simulations revealed that TC150, TC151, and TC153 bind to OXER1 (they interact with the same binding grove as 5-oxo-ETE and testosterone, not shown) and the ligand-receptor complex bound $G_{\alpha}$ with a $\Delta G$ GPCR(L)-$G_{\alpha}$-GDP $\sim$657, $\sim$645, and $\sim$635 kcal/mol, respectively, pointing out an antagonistic nature, compatible with that of testosterone. Finally, a series of polyphenols (epicatechin and its dimers B2 and B5), which we have previously reported as mimicking membrane testosterone actions, showed $\Delta G$ GPCR(L)-$G_{\alpha}$-GDP values $\sim$642, $\sim$665, and $\sim$736 kcal/mol, respectively, identifying them as antagonists (epicatechin, B2) or partial agonist (B5).

3.2.2 | Detection of agonists and antagonists for a novel receptor (OXER1)

A valid prediction method should provide useful hints about the agonistic or antagonistic properties of both crystalized or not GPCRs. Hence, we used our approach to predict the agonistic-antagonistic properties of a number of substances, of very different molecular structure (lipids, steroids, polyphenols), on the oxo-eicosanoid receptor OXER1.

OXER1 was deorphanized in 2002-3 and was found to be the endogenous receptor for the arachidonic acid metabolic product 5-oxo-ETE, produced through the action of 5-lipoxygenase (5-LOX) and peroxidase. However, recently, we have reported that OXER1, coupled to $G_{\alpha}$ protein, also mediates membrane-initiated androgen actions (see, and references herein), with testosterone acting as an antagonist. As OXER1 has not been crystallized yet, we have used the Swiss Model Biospace to provide the most promising solution of its 3D structure and retained a solution, based on P2Y purine receptor, for docking simulations.

$G_{\alpha}$-GDP bound to the 5-oxo-ETE (agonist)- or testosterone (antagonist)-OXER1 complex, with a $\Delta G$ GPCR(L)-$G_{\alpha}$-GDP of $\sim$836 and $\sim$663 kcal/mol, respectively (Table 5). We have also calculated the affinity of a series of derivatives of arachidonic acid biotransformation, which have been previously reported to act as partial OXER1 agonists (see https://geneards.weizmann.ac.il/v3/cgi-bin/carddisp.pl?gene=OXER1 and references therein). Obtained $\Delta G$ GPCR(L)-$G_{\alpha}$-GDP values are intermediate between 5-oxo-ETE and testosterone, verifying their partial agonistic nature (Table 5).

In addition to the above compounds, we have tested a series of pregnenolone analogs, with reported antiproliferative activity in different cancer cell lines. As shown in Table 5, docking simulations revealed that TC150, TC151, and TC153 bind to OXER1 (they interact with the same binding grove as 5-oxo-ETE and testosterone, not shown) and the ligand-receptor complex bound $G_{\alpha}$ with a $\Delta G$ GPCR(L)-$G_{\alpha}$-GDP $\sim$657, $\sim$645, and $\sim$635 kcal/mol, respectively, pointing out an antagonistic nature, compatible with that of testosterone. Finally, a series of polyphenols (epicatechin and its dimers B2 and B5), which we have previously reported as mimicking membrane testosterone actions, showed $\Delta G$ GPCR(L)-$G_{\alpha}$-GDP values $\sim$642, $\sim$665, and $\sim$736 kcal/mol, respectively, identifying them as antagonists (epicatechin, B2) or partial agonist (B5).

In order to verify our prediction, we have experimentally tested whether these compounds can antagonize 5-oxo-ETE action on cAMP production, like testosterone. OXER1-$G_{\alpha}$ interaction results in an inhibition of cAMP. This is experimentally tested by stimulating cAMP production in cells by forskolin and detecting the cAMP inhibition after incubation of cells with the corresponding ligands. We have previously shown that testosterone incubation of prostate cancer cells reverts the 5-oxo-ETE-induced inhibition, in a dose-dependent manner. Here, we have applied the same protocol using pregnenolone analogs and polyphenols, after forskolin stimulation of DU145 human prostate cancer cells and application of 5-oxo-ETE. Table 5 presents the normalized cAMP inhibition (5-oxo-ETE inhibition = 100%). Testosterone reverts this inhibition by 51%, at a concentration 1 $\mu$M, as reported previously. Of the tested compounds, all reverted 5-oxo-ETE cAMP inhibition by 48%-67%, at the same 1 $\mu$M concentration, classifying them as antagonists of OXER1, with the notable exception of B5 procyanidin, which reverted 5-oxo-ETE cAMP inhibition by only 25%, classifying it as a partial agonist, as also suggested by the in silico binding data.

### Table 4

Simulation data of four novel compounds approved by the FDA in 2018. For each compound its affinity (Galaxy Docking) and its interaction with the corresponding $G_{\alpha}$ protein are shown. The interaction of each drug with the GPCR analyzed here and with its cognate receptor (for which an FDA approval was provided) is shown. $X$ denotes nonassociation.

| Ligand            | Receptor                     | Galaxy Docking | HEX Docking with $G_{\alpha}$-GDP | HEX Docking with $G_{\alpha}$-GTP | Comment                        |
|-------------------|------------------------------|----------------|-----------------------------------|-----------------------------------|--------------------------------|
| Prulalopride      | 5-HT$_1B$                    | $-12.323$      | X                                 | X                                 | Selective Agonist of 5-HT$_4$ Receptor |
|                   | 5-HT$_4$ ($G_{\alpha}$)      | $-13.566$      | $-1065.67$                        | $-690.85$                        |                                |
| Lofexidine        | $\alpha_2A$-Adrenergic       | $-10.619$      | X                                 | X                                 | Selective Agonist of $\alpha_2A$- Adrenergic Receptor |
|                   | $\alpha_2X$-Adrenergic       | $-8.933$       | $-1061.31$                        | $-473.26$                        |                                |
| Latanoprostene    | PTGDR2                       | $-20.039$      | X                                 | X                                 | Selective Agonist of Prostaglandine F Receptor |
|                   | Prostaglandine F-R ($G_{\alpha}$) | $-19.614$      | $-1029.24$                        | $-551.30$                        |                                |
| Naldemedine       | $\mu$-Opioid                 | $-15.576$      | $-657.09$                         | $-594.69$                        | Opioid Receptor Antagonist     |
could provide an initial screening of chemicals as positive hits, might be of interest for the selection of interesting compounds, which could decrease the time-frame in drug discovery, prior to in vitro and in vivo validation. Here, we report a method (see Figure 1 for a schematic representation) which may be used for the initial, in silico screening of potentially active compounds, taking into account the binding of the ligand on the corresponding receptor and its subsequent simulated affinity for $G_{\alpha}$-GDP. We report that the latter may correctly discriminate >90% of substances between agonists, partial agonists and antagonists.

GPCRs-related drugs account for 34% of all drug targets.\textsuperscript{2-3,43} In addition, several pharmacological substances, designed to interact with a single target, were found to mediate effects via several GPCRs, exhibiting a specific polypharmacological profile (see\textsuperscript{12} for a discussion). However, the crystal structures of only 62 unliganded GPCRs are available today, and 206 in combination with different agonistic or antagonistic small molecules (https://gpcrdb.org/structure/statistics), whereas almost 100,000 distinct putative GPCR ligands have been reported in ChEMBL\textsuperscript{44} of them, biological activity has been reported only for only 3%. Our in silico approach, based on publicly available programs and web resources, may be used as an initial pipeline for the identification of compounds to be further tested as putative drug candidates. This was further verified here, with a noncrystalized GPCR (OXER1), on which, our pipeline correctly identified agonists and antagonists.

The novelty of our approach relies on exploiting, in addition to ligand-GPCR fully flexible docking, an initial step of the subsequent signaling event, their interaction with $G_{\alpha}$-proteins.\textsuperscript{25} to provide a quick initial estimate of ligand agonistic or antagonistic properties. In our analysis, agonistic ligands induce a significantly higher affinity for the liganded receptor $G_{\alpha}$-GDP interaction. This affinity decreases substantially when the same G-protein is bound to GTP, expressing the biologically relevant dissociation of the GTP-bound G-protein from the receptor and the initiation of intracellular signaling events.\textsuperscript{25} Our approach is based on bibliographic data from known ligand interactors of crystalized or noncrystalized $G_{\alpha_{o}}, G_{\alpha_{i}}, G_{\alpha_{s}}$ interacting receptors. The obtained solutions were compatible with biological data and correctly predict the full or partially agonistic and antagonistic properties of the ligands. Furthermore, the obtained solutions of the liganded receptor-GDP/GTP bound $G_{\alpha}$ heteroprotein complexes do not differ significantly from the corresponding crystal structures, whenever available. However, in its current form, the proposed approach has some drawbacks (not fully automated, necessitating human intervention for the selection of the $G$-protein-receptor binding solution and not taking into account GPCR-$\beta$-arrestin, $G_{\beta\gamma}$ or allosteric binding). In addition, the proposed cut-offs may be refined with the addition of additional GPCRs and ligands, or modified if other simulation programs are used for the calculation of GPCR-L and $G_{\alpha_{o}}$-GDP affinities.

### TABLE 5 Fully flexible ligand binding results on the OXER1 receptor, together with the liganded GPCR (GPCR(L)) binding to $G_{\alpha}$ in its GDP- and GTP-bound forms. All data are reported as differences in the Gibbs-free energy ($\Delta G$), expressed in kcal/mol. Data from 5-HETE, 12-HpETE, 15-HpETE, 12-HETE, and 15-HETE were from previous studies, whereas data for all other compounds were experimentally verified, through an inhibition of 5-oxo-ETE effect on cAMP production. Here, the maximum inhibition of forskolin stimulated inhibition of cAMP production by 1 $\mu$M 5-oxo-ETE (the natural ligand of OXER1 receptor) was set as 100% inhibition, and data obtained by all other compounds were compared to this maximum value at a similar 1 $\mu$M concentration, added simultaneously with 5-oxo-ETE. Please refer to the Material and Methods section, to Figure 3E and text of reference (38), and to Figure S3 for further details. The effect column presents the reported action of the compound (bibliography), the experimental validation (experimental), and the predicted effect by the proposed model (Model). See text for further details.

| RECEPTOR | LIGAND   | GPCR-Ligand (kcal/mol) | GPCR(L)-G$_{\alpha}$GDP (kcal/mol) | GPCR(L)-G$_{\alpha}$GTP (kcal/mol) | % cAMP INHIBITION (Experimental data*) | EFFECT Bibliography/ (Experimental)/ Model |
|----------|----------|------------------------|-----------------------------------|-----------------------------------|----------------------------------------|--------------------------------------------|
| OXER1    | 5-oxo-ETE| -14.5                  | -896.5                            | -528.3                            | 100                                    | Ago/(Ago/Ago)                              |
|          | Testosterone | -10.9                     | -663                              | -507.9                            | 51 ± 2.55                              | Antago/(Antago/Antago)                     |
|          | 5-HETE    | -14.5                  | -710.8                            | -565.5                            | NA                                     | PA/PA                                      |
|          | 12-HpETE  | -14.2                  | -713.8                            | -521.4                            | NA                                     | PA/PA                                      |
|          | 15-HpETE  | -14.1                  | -758.3                            | -544                              | NA                                     | PA/PA                                      |
|          | 12-HETE   | -14.5                  | -717.7                            | -566.3                            | NA                                     | PA/PA                                      |
|          | 15-HETE   | -13.3                  | -723.8                            | -566.6                            | NA                                     | PA/PA                                      |
|          | TC150     | -15.4                  | -657.3                            | -544.2                            | 48 ± 1.92                              | NA/(Antago/Antago)                         |
|          | TC151     | -16.1                  | -645.2                            | -541.1                            | 66 ± 4.81                              | NA/(Antago/Antago)                         |
|          | TC153     | -14.5                  | -635.2                            | -572.6                            | 54 ± 3.23                              | NA/(Antago/Antago)                         |
|          | B2        | -25.4                  | -665.2                            | -485.1                            | 48 ± 3.47                              | NA/(Antago/Antago)                         |
|          | B5        | -25.1                  | -736.2                            | -590.4                            | 25 ± 5.31                              | NA/(PA/PA)                                |
|          | Epicatechin | -13                     | -642.8                            | -552.5                            | 67 ± 2.98                              | NA/(Antago/Antago)                         |

Abbreviations: Ago, Agonist; Antago, Antagonist; GPCR(L), Ligand-bound GPCR; NA, Non-available; PA, Partial Agonist.

*Mean ± SE, n = 3.
5 | CONCLUSION

Our data clearly show that, by integrating sequential steps of receptor downstream signaling in ligand-GPCR simulations, as expressed by GDP-Gα_i binding, we can correctly predict the nature (agonist, antagonist, partial agonist) of a given small molecule. This approach, combined to properly implemented and successfully validated QSAR methods, may represent a useful addition to current research processes for the initial prediction and design of novel GPRC-interacting molecules. It might be of interest to explore further whether similar initial estimates might be also applied on other, non-GPCR, receptors, which could provide a generalization of our approach.

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DISCLOSURES

None declared.

AUTHORS’ CONTRIBUTIONS

MK and EC conceived and designed the study, and wrote the paper. AP, CP, KK, and PM performed the analyses and experiments. AP, TC, GN, and PAT participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

ETHICAL STATEMENT

This article does not contain any studies involving animals or human participants performed by any of the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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