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I have carefully read and evaluated the PhD Thesis of Mr. Stefan Mordalski, entitled **Computational studies on the structures of G protein-coupled receptors. Tools supporting homology modeling and the analysis of ligand-receptor interactions.**

The PhD Thesis presents the development of a diverse set of useful bioinformatics and cheminformatics tools and methods for the analysis and prediction of G Protein-Coupled Receptor (GPCR) structure and interactions with small-molecule ligands. The PhD work has resulted in two key publications reflecting the contents of chapters 3-5 and added as appendix to the Thesis: one as first author, and the other as second author, in important journals in the field of bioinformatics (*Nucleic Acids Research*) and cheminformatics (*Journal of Cheminformatics*). Furthermore at least three other publications of Mr Mordalski (in *Bioorg Med Chem Lett*, *J Chem Info Model*, *Tr Pharmacol Sci*) that are closely related to the work presented in the Thesis. In addition to these publications, several of the tools developed as part of the PhD project are accessible via GPCRdb.org, providing an excellent source for GPCR structural data mining. I congratulate Mr. Stefan Mordalski and his supervisors Prof. Dr. Andrzej Bojarski and Dr. David Gloriam with the result and **I hereby recommend to accept the PhD Thesis for oral defense by Stefan Mordalski in order to obtain the PhD degree.**

I have complemented a short review of the scientific value of the work presented in the PhD Thesis with some comments that can help Mr Mordalski to prepare for his PhD Thesis defense.

The introduction **Chapter 1** (p. 13-31) provides a review of structural analyses of G Protein-Coupled analyses and includes several Figures extracted from own publications and the publications of other groups. The chapter provides a summary of the current structural information that is available for GPCRs as well as an introduction of the bioinformatics and cheminformatics methods described in the Thesis.

The aims of the PhD Thesis are defined in **Chapter 2** (p. 32): the development of tools and methods for GPCR homology modeling, the analysis of GPCR-ligand interactions, and GPCR structure-based sequence analyses. The overall aim and objectives of the PhD Thesis indeed address important questions and challenges in the GPCR research field, and the area structural protein modeling in general.

In **Chapter 3** (p. 33-48), the methods applied during the PhD Thesis studies are described, including protein homology modeling, molecular docking, structure-based pharmacophore modeling, structure-based sequence analyses, structural interaction fingerprint analyses, and machine learning. The chapter furthermore provides an overview of the current GPCR crystal structure and GPCRdb data used for the studies presented in the Thesis.

Chapter 4 (p. 49-66) describes retrospective virtual screening studies against homology models of beta-2 adrenergic receptor based on ensembles of different GPCR crystal structure templates (Mordalski et al. (2015) *Journal of Cheminformatics* 7: 13). The study represents an interesting and valuable evaluation of homology modeling approaches, but seems to contain some discrepancies with previously reported retrospective virtual screening assessments against beta-adrenergic receptors (see detailed comments below) that can be interesting points of discussion during the oral PhD Thesis defense.

In **Chapter 5** (p. 67-74) describes the development of a new implementation of the SIFt structural interaction fingerprint 2D SIFt, combining ligand-centric and protein-centric interaction fingerprint information. The combined ligand and protein pharmacophore feature matrix seems to offer a novel way to encode protein-ligand interactions in 7 x 9 unique interaction fingerprints. The 2D SIFt examples however only contain discrete matched combinations of ligand and protein features, resemble the previously reported 1D structural interaction fingerprints (see Detailed comments Chapter 5). In addition structure-based sequence alignment and pharmacophore mapping tools are described that have been made available via GPCRdb.org and are presented in a high impact publication (Isberg, Mordalski et al. (2015) *Nucleic Acids Research* 44: D356).

Chapter 6 (p. 74-75) provides a short overview of conclusions regarding the presented homology modeling, structural interaction fingerprint analysis, pharmacophore fragment mapping, and structure-based sequence alignment methods.

Comments Chapter 1:

1) On page 23 it is stated that "the crystal structures of rhodopsin which were solved using peptides derived from transducin (Gt) and β -arrestin (PDB: 3PQR and 3DQB, respectively), reveal nearly identical conformations of those peptides (Fig. 10).", which is later repeated in the caption of Figure 10 itself. PDB ID 3DQB however does *not* contain a crystal structure of rhodopsin in complex with a β -arrestin derived peptide, but contains a structure of rhodopsin in complex with a C-terminal peptide derived from transducin (Gt). On page 24 it is stated that there are "*crystal structures of the GPCRs solved using (...) β -arrestin (4PXF)*", but this is in fact a peptide derived from β -arrestin, while an arrestin bound rhodopsin crystal structure has been deposited under PDB ID 4ZWI.

Comments Chapter 3:

2) Based on the examples provided in Table 7 and Figure 18 I cannot really figure out what added level of detail the 2D annotation of ligand-centric and protein-centric interaction features in 2D SIFt would bring compared to the 1D annotation of specific interaction types between specific features in the ligand and protein. Moreover, it seems that the 2D SIFt example presented in Figure 18 gives room for inconsistent annotation of interactions that may complicate efficient comparison of the interaction fingerprints for e.g. docking pose analysis and virtual screening. The 7 bit IFP described by Marcou and Rognan for example includes:

- Bit 1: Non polar contact, to some extent comparable to similar to Hydrophobic or VdW in SIFt, indicated in the example in Table 7.
- Bit 2 and bit 3: Aromatic face-to-edge and aromatic face-to-face interactions, respectively, which in principle provides more detail on the character of the aromatic protein-ligand pi-pi interaction than the single R feature in SIFt (see e.g. the R ligand feature x R protein feature interaction for F^{6,52} in Fig. 18).
- Bit 4: H-bond donor (ligand) to H-bond acceptor (protein residue) interaction, which is annotated in the same way in the 2D SIFt example in Table 7 (D ligand feature x A protein feature).

- Bit 5: H-bond acceptor (ligand) to H-bond donor (protein residue) interaction, which would similar to the A ligand feature x D protein feature in 2D SIFt.
- Bit 6: Interaction between positively charged group (ligand) to negatively charged group (protein residue), annotated in the 2D SIFt interaction with D^{3.32} in Fig. 18, although the 2D SIFt protein *Charge* feature apparently does not discriminate between a positive or a negative charge (why not?).
- Bit 7: Interaction between negatively charged group (ligand) to positively charged group (protein residue)

This comparison raises several questions that I hope the candidate can show the added value of the 2D SIFt method over 1D interaction fingerprint methods as stated in Chapter 5:

- i) Why is the interaction feature of D^{3.32} in the 2D SIFt in Fig. 18 annotated as polar (P) and not as a H-bond acceptor (A) that interacts with the H-bond donor (D) of carazolol (in line with 1D IFP)?
- ii) Why does the 2D SIFt protein *Charge* feature does not discriminate between a positive or a negative charge (in line with 1D IFP)?
- iii) Does the 2D SIFt distinguish 7 ligand features x 9 protein residue features = 63 different interaction fingerprints? Does 2D SIFt for example in addition to interactions between matching ligand and protein pharmacophore features also encode “interactions” or “mismatches” (i.e. contacts within a certain cutoff distance) between a ligand hydrophobic feature and a protein H-bond donor feature? Or does it in fact only allow distinct, matching ligand and protein residue interaction features, like in fact the 1D interaction fingerprints described above?

A more comprehensive description and justification of the 2D SIFt approach, including a clear comparison with 1D interaction fingerprint schemes, will be very much appreciated.

3) There are errors and inconsistencies in the analyses of the interactions between carazolol and β_2 -AR in Figure 16 and between BI-167107 and BI-167107 in Figure 19 (which may have implications on the accuracy of the SIFt analyses presented in Chapter 5). The interaction fingerprint in Figure 16 indicates an H-bond donor (to H-bond acceptor) interaction between the hydroxyl group of carazolol and the carboxylate oxygen of D^{3.32}, but this is not consistent with the H-bond interaction geometry (distance/angle) between the H-bond donating hydroxyl oxygen, the donated hydroxyl hydrogen atom, and the H-bond accepting carboxylate oxygen. In Figure 19 the hydroxyl group of BI-167107 is supposed to donate an H-bond to the other carboxylate oxygen of D^{3.32} as well, but the position of the hydroxyl hydrogen is apparently not optimized either in the presented protonated β_2 -AR structure. In addition an alternative H-bond network between BI-167107 and S^{5.42x43} and S^{5.46} can be envisioned that satisfies a more extensive H-bond interaction network than the one proposed in Figure 19, in which for example: i) the amide nitrogen of BI-167107 donates a H-bond to the hydroxyl oxygen of S^{5.42x43}, ii) the hydroxyl group of S^{5.42x43} donates a H-bond to the hydroxyl oxygen of BI-167107, iii) the hydroxyl group of BI-167107 donates a H-bond to the hydroxyl oxygen of S^{5.46}, and iv) the hydroxyl group of S^{5.46} donates a H-bond to the backbone carbonyl oxygen of S^{5.42x43}.

These inaccuracies invoke important questions about the consistency and accuracy of the fragment mapping procedure presented in Fig. 19, as well as the protein preparation procedure used to derive GPCR-ligand 2D SIFt interaction fingerprints described in Chapter 5 (see also detailed comments Chapter 5). Could the candidate please more carefully explain how the topology and protonation of the ligand was curated and checked and how e.g. protein-ligand H-bond interaction networks were evaluated to derive robust pharmacophore interaction maps and 2D SIFts described in Chapter 5?

4) Table 9 presents 4 antagonist bound and 5 agonist bound crystal structures of β_2 -AR, but agonist bound (3P0G, 3PDS, 3SN6) and one antagonist bound (4GBR) β_2 -AR structures are missing. Consideration of these structures as well as the 9-11 antagonist bound and 5 agonist bound crystal structures of the homologous β_1 -AR (which have a similar functional effect on β_1 -AR) would have enabled a more robust comparison and analysis of structural interaction patterns of agonist vs.

antagonist with beta adrenergic receptors (as described for example in Kooistra et al. (2015) *J Chem Info Model* 55: 1045). A discussion of the limited scope of the evaluation would therefore in my opinion be appropriate.

Comments Chapter 4:

5) The MCC values for β_2 -AR are surprisingly low (Figure 1) given the high virtual screening accuracies obtained in earlier VS studies against beta-2 adrenergic receptor, including e.g. de Graaf et al. (2008) *J Med Chem*, Kooistra et al. (2015) *J Chem Info Model*, and Weiss et al. (2016) *J Chem Info Model*. The fact that only a MCC measure is used to assess virtual screening accuracy (instead of the commonly used enrichment at 1% or 5% false positive rate or area under the ROC curve measures) unfortunately does not allow direct comparison with these studies, but the MCC value below 0.5 presented in chapter 3 for example is not in line with the 28 fold enrichment at 1% EF obtained by Weiss et al. who used the same docking program (Glide) and a similar data set (e-DUD). Can this discrepancy with previous reports be explained?

6) Table 4 indicates that the number of actives and inactives are increased by almost 100% in the LigPrep step, whereas DUD and ZINC data sets are increased by only about 25%. The generation of different protonation, tautomers, and/or isomers is in principle justified when these are indeed not defined for the actives/inactives/decoys (although I believe that in the ZINC and DUD data sets different protonation and tautomer states have in fact already been defined)? The consideration of different protonation states/tautomers/stereoisomers (generated from the same original compound) as different compounds in the assessment of the virtual screening enrichments is however *not* in line with a real life prospective virtual screening study in which different forms of the same compound would be considered as a single hit for experimental validation. A careful evaluation how these inaccuracies and inconsistencies would affect the (limited) differences observed in virtual screening results between different models (10% differences in MCC) is therefore in my opinion required.

Comments Chapter 5:

7) Several relevant publications on (other) structural protein-ligand interaction fingerprint methods and applications are unfortunately missing in this chapter, including the methods described by Cao et al. (2015) *ChemMedChem* (LIFT), Sato et al. (2014) *J Chem Info Model* 54: 3153 (PLIFs), Kireev et al. (2014) *J Chem Info Model* 54: 2555 (SPLIFs), and Desaphy et al. (2013) *J Chem Info Model* 53: 623 (TIFP). Discussion of the retrospective virtual screening studies described in Kooistra et al. (2015) *J Chem Info Model* 55:1045 and de Graaf et al. (2008) *J Med Chem* 51: 4978 will be relevant as well as these studies focus on the discrimination of beta adrenergic agonists vs. antagonists, which is the focus of the Chapter. Furthermore it should be noted that interaction fingerprints are not only integrated in Schrodinger, but also part of for example ICM-Pro and MOE modeling software. A more careful comparison to other interaction fingerprint approaches is in my opinion required to more accurately assess the scientific value of the 2D SIFt methodology method described. The limited references and comparison to other interaction fingerprint methods requires a justification in my opinion.

8) There are unfortunately many errors in the interaction analysis presented in Table 11:

- R^{3.28} does not accept an H-bond, but donates an H-bond donor to the ligand in S1PR1 (3V2Y) and should therefore be colored blue, not yellow
- D^{3.32} forms an H-bond to the co-crystallized ligand in the H₁R (3RZE), 5-HT₁BR (4IAR), and 5-HT₂BR (4IB4) and should therefore be color coded yellow (like in e.g. β_1 -AR (4BVN), β_2 -AR (2RH1), D₃R (3PBL), M₂R (3UON) receptors) instead of grey (any interaction).
- R^{3.33} donates an H-bond to the ligand in P2Y₁₁ (4PXZ, not 4PZX as incorrectly indicated in the figure) and should be colored blue (instead of the aromatic green color).

- K^{5.39} (erroneously indicated as 5.40?) does not accept an H-bond with the ligand in the kappa opioid receptor (4DJH) and should therefore not be color coded yellow.
- F^{6.52} forms similar aromatic interactions with the ligands in beta-2 adrenergic receptor (2RH1) and H₁R (3RZE), but only the β_2 -AR (2RH1) entry is colored orange.
- R^{7.35} (erroneously indicated as 7.34 which is not directed towards the binding site?) does not accept an H-bond, but donates an H-bond donor to the ligand in FFAR1 (4PHU) and should therefore be colored blue, not yellow.
- E^{7.39} (erroneously indicated as 7.38 which is not directed towards the binding site?) forms an H-bond to the co-crystallized ligand in CCR5 (4BMS) and CXCR4 (3ODU) and should therefore be color-coded yellow instead of grey (any interaction).

Furthermore the structural analysis does not seem to allow the annotation of multiple interaction types for the same residue, e.g.:

- N^{6.55} accepts an H-bond (yellow) and donates an H-bond (blue) to the ligand in the A_{2A}, but the H-bond donor (blue) is not shown?
- N^{7.39} (erroneously indicated as 7.38?) accepts an H-bond (yellow) and donates an H-bond (blue) to the ligand in β_2 -AR (2RH1), but the H-bond donor (blue) is not shown?

Finally it should be noted that for several receptors multiple (antagonist bound) structures are available (not only β_1 -AR (11) and β_2 -AR (9), but also A_{2A} (9), CXCR4 (6), mouse/human δ opioid (4), human/rat M₃R (4), LPA₁ (3), P2Y₁ (2), AT₁ (2), 5HT_{1BR} (2) and therefore the limited selection of only one single antagonist bound structure per receptor in Table 11 gives a limited view. Can the candidate provide a justification for this limitation and indicate how this limitation affects the presented analysis?

9) A reference to Figure 30 to support a statement on the application of 2D SIFt for virtual screening seems misplaced: *“The main purpose of the new fingerprint is its use for VS, and the initial experiment demonstrates (Fig. 30) the superiority of 2D-SIFt, which slightly outperforms ligand-based methods in the active/inactive classification.”* Figure 30 however shows a *“Screenshot of the interactive crystal structure browser tool providing access to alignment, superposition and download of the available crystal structures of the GPCRs.”* and the supposed proof that 2D-SIFt is superior in virtual screening studies (which is repeated in the conclusion Chapter 6) appears to be lacking.

Comments Chapter 6:

10) One of the statements claims that there is per definition a discrepancy between VS accuracy and ligand binding mode prediction accuracy: *“In terms of VS the interaction based approach relies on the differences between the interactions of active and inactive compounds rather than on the biological soundness of the complexes. This feature results in significant differences between receptor models intended for use in virtual screening and those focused on providing an explanation for SAR or the molecular mechanism of binding.”* This supposed disconnection between binding mode prediction and virtual screening is not in line with for example my own experiences with virtual screening studies using interaction fingerprints to identify GPCR ligands with specific functional effects (Kooistra et al. (2015) *J Chem Info Model* 55:1045, Kooistra et al. (2016) *Sci Rep* 6: 28288) and GPCR structure-based optimization, SAR, and mutagenesis studies of structure-based virtual screening hits (de Graaf (2011) *J Med Chem* 54: 8195, Kuhne et al. (2016) *submitted to J Med Chem*).

Yours sincerely,



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