JOURNAL OF CHEMICAL INFORMATION AND MODELING

Article

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On-the-Fly Integration of Data from a Spin-Diffusion-Based NMR Experiment into Protein−Ligand Docking

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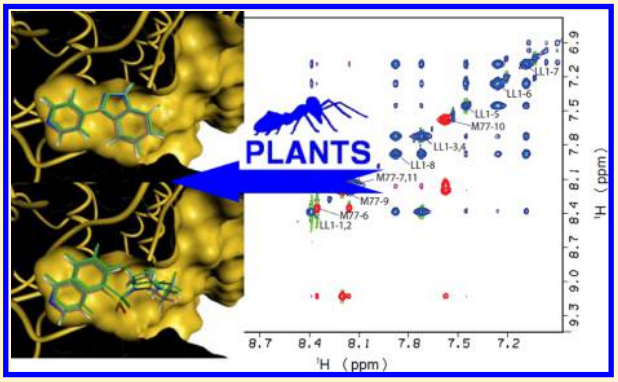
Supporting Information

ABSTRACT: INPHARMA (interligand nuclear Overhauser enhancement for pharmacophore mapping) determines the relative orientation of two competitive ligands in the protein binding pocket. It is based on the observation of interligand transferred NOEs mediated by spin diffusion through protons of the protein and is, therefore, sensitive to the specific interactions of each of the two ligands with the protein. We show how this information can be directly included into a protein−ligand docking program to guide the prediction of the complex structures. Agreement between the experimental and back-calculated spectra based on the full relaxation matrix approach is translated into a score contribution that is combined with the scoring function ChemPLP of our docking tool PLANTS. This combined score is then used to predict the poses of five weakly bound cAMP-dependent protein kinase (PKA) ligands. After optimizing the setup, which finally also included trNOE data and optimized protonation states, very good success rates were obtained for all combinations of three ligands. For one additional ligand, no conclusive results could be obtained due to the ambiguous electron density of the ligand in the X-ray structure, which does not disprove alternative ligand poses. The failures of the remaining ligand are caused by suboptimal locations of specific protein side chains. Therefore, side-chain flexibility should be included in an improved INPHARMA-PLANTS version. This will reduce the strong dependence on the used protein input structure leading to improved scores overall, not only for this last ligand.



INTRODUCTION

Ligand-based spin diffusion NMR techniques, such as transferred nuclear Overhauser enhancement (trNOE),[1] saturation transfer difference (STD),[2,3] difference of inversion recovery rate with and without target irradiation (DIRECTION),[4,5] waterLOGSY,[6,7] and interligand NOEs for pharmacophore mapping (INPHARMA),[8−11] provide valuable information on the parts of the ligands involved in binding to the protein, the ligands bound conformations, and the relative orientation of two ligands in the binding site, respectively. In this way, they can be used as alternative to X-ray crystallography to determine protein−ligand complex structures, especially in cases where suitable crystals cannot be obtained. To reach their full potential, NMR techniques are combined with theoretical approaches, such as docking, whereby docking poses are evaluated by comparing experimental and back-calculated NMR parameters. Docking methods have been optimized to generate a large number of ligand poses in the binding site of the protein, to rank these using very simple scoring functions, and finally to filter out the most promising ones. Due to the simplicity of the scoring functions, the fine energetic aspects of the binding cannot be described exactly and false positives, i.e. poses very different to the experimentally determined structures, often have very similar or even higher scores than the correct one. If a ligand is redocked into its native structure, i.e. the X-ray structure of the protein in complex with the same ligand, state-of the-art docking tools can obtain success rates of over 80%,[12−15] where success is defined as a best-ranked pose with a heavy-atom root-mean-square deviation (RMSD) to the X-ray structure of less than 2 Å. Docking failures, however, increase drastically when the apo structure (protein structure without any ligand) or a structure with another ligand (cross-docking) is used. Here, the data from the NMR experiments can remove false positives and unambiguously identify the correct pose. NMR chemical shift perturbations (CSP) have

Received: May 15, 2015
Published: July 30, 2015

DOI: 10.1021/acs.jcim.5b00235
J. Chem. Inf. Model. 2015, 55, 1962−1972

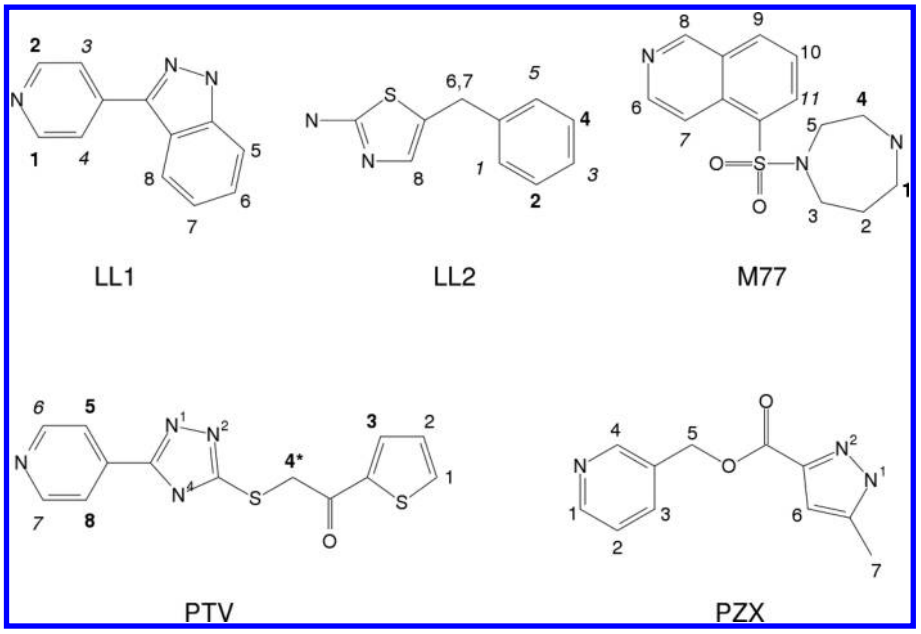


Figure 1. Five PKA ligands used as validation set for the INPHARMA docking. Numbers mark the protons visible in the NMR experiment.

often been used to guide docking calculations. The first quantitative treatment of CSPs was implemented in the HADDOCK approach for protein−protein docking and successfully applied to a number of systems.[16−18] König et al.[19] as well as McCoy and Wyss[20] additionally included residual dipolar couplings. Protein−ligand docking methods including CSPs were published e.g. by Cioffi et al.,[21] Wang et al.,[22] Gonzalez-Ruiz and Gohlke,[23] and the group of Krimm.[24,25] Even if CSPs are very sensible and, thus, provide a large amount of information, they have the disadvantage that they are not always caused by direct ligand interactions but by changes in the conformation or the dynamics of the protein upon binding and can occur far away from the actual binding site.[24,25] STD data can be used in a qualitative way[26] or quantitatively based on the CORCEMA approach.[27,28] In a recent publication, Skjaerven et al.[29] demonstrated that INPHARMA is able to correctly predict the poses of the five ligands shown in Figure 1 in the active site of the cAMP-dependent PKA (two representative INPHARMA spectra are shown in the Supporting Information). For this, information from multiple INPHARMA experiments was combined according to the proposed INPHARMA-STRING method.[29] It follows a three-step procedure. First, a large number of poses is generated for each ligand using a standard docking tool. Then, a pose for each ligand in a specific pair is chosen and the relative orientation of the two ligands is rescored by comparing the signals in the INPHARMA spectrum with the back-calculated theoretical signals. This is repeated for all possible pairs of poses, resulting in a ranked list for each ligand pair. By identifying consensus poses, which are in accordance with all experiments, i.e. they rank high with respect to the INPHARMA data in all ligand pair lists, success rates could be highly increased, even when homology models from distant homologues or structures from molecular dynamics simulations were used. Another way of combining data from different experiments has recently been described,[30] in which STD, transfer NOE, and INPHARMA data can be combined and yield more robust scoring results for pairs of ligands or multiple ligands than INPHARMA alone.

In this paper, we will take another route: We will try if the poses of a pair of ligands can be improved by including the INPHARMA data in the docking process. For the effective back calculation of INPHARMA spectra, the spINPHARMA package was developed and is now publicly available.[31] Briefly, spINPHARMA calculates, based on a complete relaxation matrix approach, the expected NOESY peaks for multiple ligand/protein complexes under exchange. Complete relaxation matrix calculations are required for INPHARMA since it involves at least a two step "spin-diffusion" transfer to obtain cross peaks between two ligands that bind consecutively to the same pocket. In this way, it can be used to evaluate a pair of ligand poses by comparing back-calculated and experimental spectra in a stand-alone version but also as a library, which can be included in other programs using a standardized interface. With this interface, it was possible to include the NMR data on the fly in the docking program PLANTS developed in our group. PLANTS is based on ant colony optimization (ACO),[32] in which the computational problem is translated into a path searching problem. Colonies of a specific ant species are extremely efficient in finding the shortest path between their nest and a food source. This is achieved by indirect communication using pheromone deposition. In the same way, the artificial ants of PLANTS deposit pheromones on the solution components of highly scored ligand poses, which then will be used more often in later iterations of the algorithm. To rank poses, the ChemPLP scoring function, specifically developed for PLANTS but now also a standard scoring function of the well-known program GOLD,[33,34] is used. It evaluates contributions from hydrogen bonding, hydrophobic and steric interactions, intramolecular clashes, as well as interactions to metal ions and essential water molecules. In the approach described here, ChemPLP is combined with scores evaluating the agreement between experimental and back-calculated NMR spectra to include the experimental data in the prediction of the poses for the five ligands from Skjaerven et al.[29]

DOI: 10.1021/acs.jcim.5b00235
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MATERIALS AND METHODS

SpINPHARMA-PLANTS Interface. The docking program PLANTS and the ChemPLP scoring function have already been described in detail elsewhere.[35,36] Therefore, we will only recapitulate the parts important for the INPHARMA implementation. Since the contributions from ChemPLP and the experimental data to the rank of a specific pose can be regarded as independent, the parameters of the ChemPLP scoring function were set to their standard values as optimized on a large test set of protein−ligand complexes.[36] However, we saw problems in the convergence of the docking runs as described below. To overcome these, some of the parameters of the ACO were modified in some of the calculations. For a standard PLANTS run, the solution components, i.e. the independent variables, are the rotational and translational degrees of freedom as well as rotatable torsion angles of the ligand. Protons of the protein able to act as hydrogen-bond donors are also treated flexible to allow for the optimization of the hydrogen-bond geometries. Each ant generates a possible solution in each iteration of the algorithm by assigning one specific value to each of these degrees of freedom according to the pheromone distribution. Better sampling of the conformational space can therefore be achieved by using a larger ant colony. Normally, 20 ants are sufficient but we also tried 50 ants to get better convergence especially for larger ligands with more degrees of freedom. The number of iterations performed by the ACO is scaled by the number of degrees of freedom of the specific complex.[36] This number can additionally be scaled by the parameter σ. Smaller values are used e.g. in virtual screenings for higher throughput. Larger values are beneficial if sampling problems are seen. Therefore, we increased it from the standard values of $\sigma = 1.25$ to $\sigma = 1.50$. Increasing it even more was not possible due to the computational demand of the back calculation of the INPHARMA spectra.

To be able to use the INPHARMA data inside PLANTS, two modifications were needed. First, the poses of two ligands have to be optimized simultaneously. Thus, both ligands have to be read in and processed. The variables to optimize are the combined degrees of freedom of both ligands. Second, for the evaluation of the poses, both ligands have to be scored independently by ChemPLP. Additionally, a score for the agreement between the experimental and back-calculated INPHARMA spectrum has to be added. The theory of such back-calculations based on the full-relaxation-matrix approach was described in great detail in the literature[8,9,37] and, therefore, will not be recapitulated here. As described in the introduction, the program package spINPHARMA was developed for the quantitative interpretation of INPHARMA data using full relaxation matrix approaches for multiple protein−ligand complexes.[31] Beside tools to calculate INPHARMA, trNOE, and STD spectra, it also contains an interface, which can be incorporated into other programs. In this way, poses can now be evaluated in PLANTS by a combination of the ChemPLP, an INPHARMA and a trNOE score, where the two latter are provided by spINPHARMA.

Combined Score for Pose Evaluation and Its Parameterization. As a measure for the agreement between the experimental INPHARMA spectrum and the one back-calculated by the spINPHARMA library, the Pearson correlation coefficient $r_{INPHARMA}$ of calculated versus experimental peak volumes is used. Since PLANTS is minimizing the scoring function, we calculate the INPHARMA score $S_{INPHARMA} = 1 - r_{INPHARMA}$. In this way, a value of 0 ($r = 1$, perfect correlation) corresponds to an optimal agreement between experimental and theoretical spectrum, 1 ($r = 0$, completely uncorrelated data) to no relationship between them (no agreement), and 2 ($r = -1$, perfect anticorrelation) to cases where large experimental volumes are correlated to small theoretical volumes and vice versa (disagreement). Since in the uncorrelated case, parts of the molecules could still be orientated correctly but in the anticorrelated case essentially all distances are wrong, it is reasonable to disfavor the latter more by the higher INPHARMA score. Similarly, a trNOE score $S_{trNOE} = 1 - r_{trNOE}$ can be defined that evaluates the agreement between experimental and back-calculated intraligand NOE signals. While INPHARMA gives information on the relative orientation of the two ligands, intraligand distances can be derived from the trNOE signals constraining the ligand conformations. Two possibilities to include the NMR data in on-the-fly pose evaluation were tested. The first one takes only the INPHARMA data into account.

$$S_1 = S_{ChemPLP,ligandA} + S_{ChemPLP,ligandB} + w_1 S_{INPHARMA}$$

The second one also includes the information from the trNOE signal.

$$S_2 = S_{ChemPLP,ligandA} + S_{ChemPLP,ligandB} + w_1 S_{INPHARMA} + w_2 S_{trNOE}$$

w_1 and w_2 are weighting factors, which are used to set the relative importance of the different parts. For the PKA system investigated here, values of 250 and 50 were identified in pretests as suitable for w_1 and w_2, respectively. Changing these weights by ±30% had only a very minor influence on the results when taking the stochastic nature of PLANTS into account (data not shown). With smaller values, the influence of INPHARMA on the docking results becomes negligible since the score is completely dominated by ChemPLP. Larger values lead to severe sampling problems. The latter is especially the case for the trNOE weight, which prohibits the theoretically more reasonable use of equal weights for INPHARMA and trNOE used e.g. in the STI approach.[30] A more detailed parametrization leading to generally applicable INPHARMA-ChemPLP scoring function will be performed on a larger and more diverse test set when this is available. For ChemPLP the standard settings as described in ref 36 were adopted. The standard search parameters like pheromone update and evaporation rates were also kept for the ACO. However, since sampling problems were seen in docking runs with two ligands, we partly modified the parameters to allow for more sampling. For these extended runs, 50 ants and $\sigma = 1.5$, with which the number of ACO iterations is controlled, were applied in contrast to the standard values of 20 ants and $\sigma = 1.25$. For each pair of ligand and each setup (ChemPLP-only, INPHARMA, INPHARMA+trNOE), 6−10 independent docking runs were performed. This provides additional sampling due to the stochastic nature of ACO. In the results, we will base the discussion on the poses with the best score from all these runs if not otherwise stated. Results for individual runs are available in the Supporting Information. Even if the standard version of PLANTS can include flexible side chains, this cannot be used for INPHARMA docking at the moment since both ligands interact with an identical protein structure. For a reasonable consideration of side-chain flexibility, these side chains have, however, to be able to adapt to both ligands

DOI: 10.1021/acs.jcim.5b00235
J. Chem. Inf. Model. 2015, 55, 1962−1972

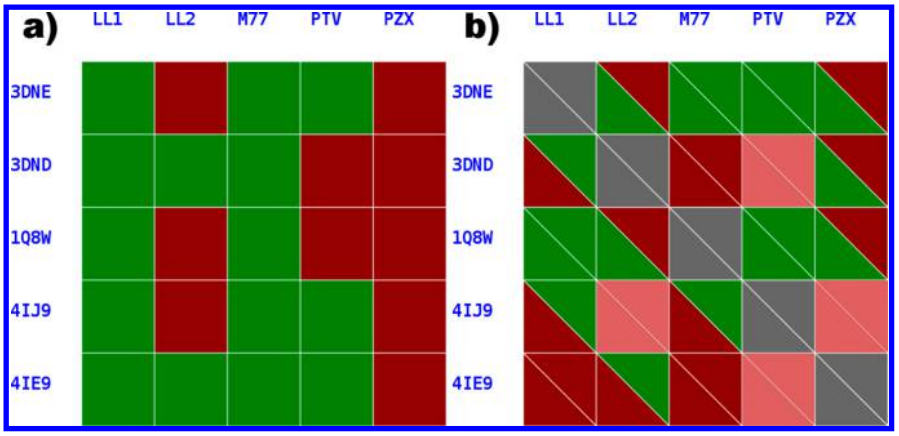

Figure 2. (a) Heat maps of the successes for the ChemPLP-only docking experiments. Here and in all following figures, each column groups the results of one ligand and each row corresponds to a protein structure taken from the complex in the specific PDB entry. Green and red: best-ranked pose has an RMSD less and more than 2 Å in comparison to the corresponding crystal structure, respectively. (b) Heat maps of the successes for the INPHARMA docking experiments (score S_1) using the original setup. The lower and upper triangle in each square corresponds to the native and the cross-docked ligand, respectively. Pairs with insufficient experimental data (see below) are shown in lighter colors.

independently, which will be possible in an improved PLANTS version currently under development.

Structure Preparation. The preparation of the input structures follows the procedure already adopted in Skjaerven et al.[29] The X-ray structures of PKA with the five ligands, 3DNE (LL1), 3DND (LL2), 1Q8W (M77), 4IJ9 (PTV), and 4IE9 (PZX) were taken from the Protein Data Bank (PDB).[38] INPHARMA requirement of common atomic nomenclature and order among the structures is fulfilled by generating homology models (Modeler Sali lab[39]) of the downloaded structures using the pdb files as templates and a common PKA sequence as query. The structures hereby generated are subsequently protonated using the AMBER software package.[40] This package is also used to generate sensible charges for the ligands. Ligand preparation is performed using the software ligprep form Schroedinger software.[41] After ligand and receptor preparation, a short minimization of the complex is made using the AMBER package, in order to avoid steric clashes. During the homology generation (which is performed without the ligand), side chains are partly moved out of their optimal position for ligand binding. As will be shown below, the minimization is not able to recover these interactions so that even the redocking experiments described below do not use the optimal protein conformation. However, we think that this is a more realistic test of the procedure since the correct protein structure is not known in real-world application predicting new complex structures.

RESULTS AND DISCUSSION

Docking Success Using Standard Setup. As described in the introduction, it was possible to elucidate the correct poses of five PKA ligands (see Figure 1) using information taken from INPHARMA measurements.[29] For this, ligand poses were generated first using a standard docking tool followed by a rescoring with INPHARMA. We will use here the same 10 INPHARMA experiments corresponding to all pairwise combinations of the ligands to validate the new approach described here. In contrast to the previous work, the agreement between experimental and back-calculated spectra is directly utilized in the docking. In the first experiments, the same setup as described in the previous publication[29] regarding the used conformations and protonation states of the protein and ligands were taken. To evaluate the performance of PLANTS on this data set, we first performed all redockings and cross-dockings using the ChemPLP scoring function[36] only, i.e. docking of a specific ligand into the protein structure native for this ligand (redocking) but also into all the other four apo protein structures (cross-docking), respectively. The results for the 25 possible experiments (5 ligands × 5 protein structures) are given as heat maps in Figure 2a, and the root-mean-square deviations (RMSDs) to the X-ray structures are in Table S1 of the Supporting Information. The ChemPLP scoring function performs well on this set with a redocking success rate of 80% (diagonal entries in Figure 2a). Only PZX cannot be predicted correctly. Also the cross-docking performance is reasonable (off-diagonal entries). LL1, M77, and PTV can be docked with an RMSD below 2 Å into all the protein structures, and LL2 can be docked into two structures. In the protein structures 3DND and 1Q8W, the central part of PTV adopts an orientation rotated by 180°, and thus, the prediction can be regarded as suboptimal. LL2 is orientated completely differently by interchanging the position of the two aromatic rings in protein structures 3DNE, 1Q8W, and 4IJ9. Both these phenomena will be discussed in detail below. Another interesting fact is that, even if PZX cannot be docked into its native structure, all other ligands can. This fact, combined with the failure of PZX in all the protein structures, suggests that the specific interactions of this ligand are very hard to predict correctly using the ChemPLP scoring function only and that the failure is not caused by a specific problem in the X-ray structure 4IE9.

We then tried if the inclusion of the INPHARMA data can improve the pose prediction especially for PZX. Since two ligands are needed for INPHARMA, we always used the ligand native to the specific protein structure (native ligand) in combination with another ligand (cross-docked ligand). The results are again summarized as a heat map (see Figure 2b) and represent the best scored poses from 16 runs (with standard and extended search settings). At the first glace, they seem to be very disappointing with only four successfully predicted ligand pairs. Correct predictions, i.e. docking poses of both ligands with RMSDs below 2 Å, could only be obtained for the pairs LL1/M77 and LL1/PTV in protein structure 3DNE as well as M77/LL1 and M77/PTV in protein structure 1Q8W. In

DOI: 10.1021/acs.jcim.5b00235
J. Chem. Inf. Model. 2015, 55, 1962−1972

all other examples, at least one ligand has a larger RMSD. Even if these results are in agreement with the publication by Skjaerven et al.,[29] where the correct prediction of all ligands was only possible by combining multiple experiments using the INPHARMA-STRING method, we will discuss in the following why including INPHARMA directly in the docking process has seemingly a deteriorating effect and how this can be circumvented by optimizing the docking setup. A few specific pairs of ligands will be first used to highlight the caveats; subsequently, the docking experiments will be repeated for all ligands with an improved setup.

LL1−PTV: Constraining the Ligand Conformation. INPHARMA dockings to predict the pair LL1−PTV in the 4IJ9 protein structure placed PTV far outside the binding site as seen in Figure 3. ChemPLP clearly favors poses close to the X-ray structures, as demonstrated by the docking success for both ligands in the ChemPLP-only runs. INPHARMA slightly favors the wrong pose leading to very comparable scores S_1 for both, the correct and incorrect PTV pose (−105.25 and −109.44, respectively). Therefore, this docking failure is caused by scoring problems, probably due to the suboptimal protein conformation as discussed in more detail below, and not by sampling problems. The fact that INPHARMA identifies the correct poses in a set of similarly ranked false positives had been already described in Skjaerven et al.[29] Nevertheless, these multiple local optima make it also harder to obtain convergence of the sampling algorithm since the search can get trapped in a local minimum. This can be seen by the very different poses obtained by independent docking runs and finding the global optimum can only be guaranteed by a larger number of such runs. Additionally, since different optima have to be exploited, less time is available to intensify the search in a specific promising region of the search space. We predict that additional information on the system would be beneficial to remove this combined scoring/sampling problem. In the INPHARMA experiments intraligand trNOE signals are also obtained. By correlating these including the diagonal peaks and their build-up rates when using multiple mixing times, information on the ligand conformation and its protein surroundings are obtained. When we include this additional information using the score S_2, the correct poses of both LL1 and PTV can be identified unambiguously (see Figure 4). In Figure 5, an overlay of the PTV conformations from the INPHARMA and INPHARMA-trNOE dockings are shown highlighting the twisting between the pyridine and the triazole ring system as well as the differences in the 2-thiophenyl ethanone part, which combined lead to the better agreement of the INPHARMA-trNOE pose with the trNOE data.

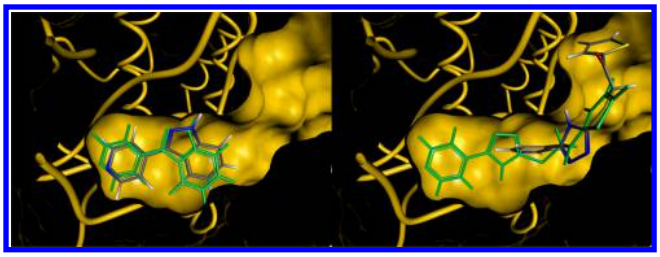

Figure 3. Best scored poses of the pair LL1 (left) and PTV (right) based on the score S_1 incorporating data from INPHARMA signals. In this and all following figures (if not explicitly stated otherwise), the docking and X-ray pose are shown in atom color and green, respectively. The LL1 pose superimposes very well with the X-ray structure, while this is not the case for PTV.

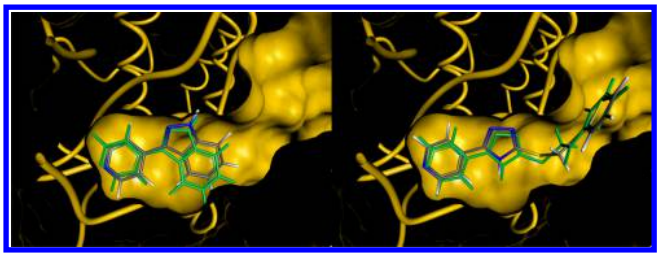

Figure 4. Best scored poses of the pair LL1 (left) and PTV (right) based on the score S_2 incorporating data from INPHARMA and trNOE signals. The false positive pose of PTV obtained based on score S_1 can be removed by restraining the ligand conformation. Poses for both ligands agree now with the X-ray structure.

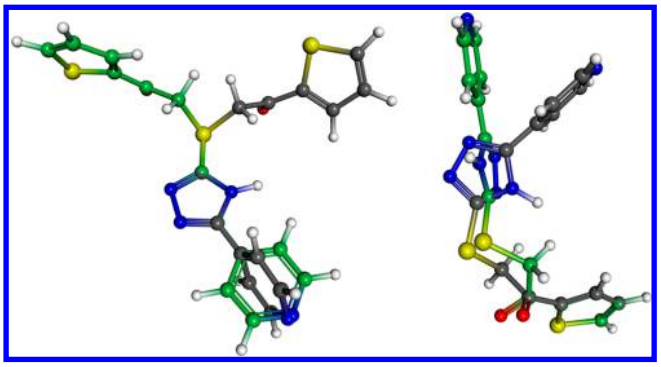

Figure 5. Overlay of the best ligand pose using INPHARMA (gray carbon atoms) and INPHARMA-trNOE (green carbon atoms) data. For alignment, the triazole (left) and thiophene (right) ring system were used, respectively, to highlight the conformational changes at both sides of the ligand.

M77−PTV: Protonation States and Suboptimal Side Chain Orientation. Intraligand information obtained from the trNOE signals of the INPHARMA experiments also improved the results for the ligand pair M77−PTV if the protein structure from PDB entry 4IJ9 is taken (both ligands were already predicted correctly using the original setup in the protein structure 1Q8W native for M77). For both ligands, RMSD values well below 2 Å are obtained. When looking at the pose of PTV, one, however, notices that the central triazole ring is flipped (Figure 6). This leads to a distortion of the hinge-region-binding pyridine. A very similar pose was also obtained by Skjaerven et al.[29] using the INPHARMA rescoring approach. In the crystal structure, the triazole shows short contacts to oxygen atoms in Thr183 and Asp184. This is compatible with

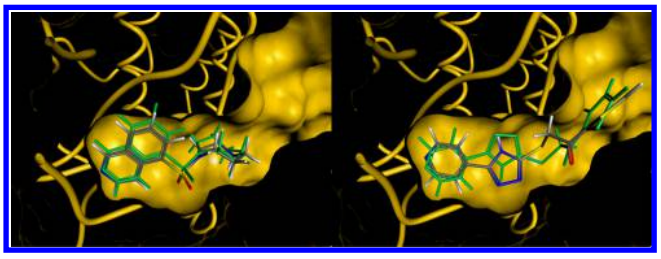

Figure 6. Poses of the pair M77 (left) and PTV (right) based on the score S_2 incorporating data from INPHARMA and trNOE signals. Due to the wrong protonation of the triazole ring system, this ring is flipped by 180°.

DOI: 10.1021/acs.jcim.5b00235
J. Chem. Inf. Model. 2015, 55, 1962−1972

two hydrogen bonds, which can be formed if the triazole is protonated at the N^1 (1H form, labeling according to Figure 1) or N^2 (2H form) but not at the N^4 (4H form) position as in the original setup. In the first case (1H form), which is proposed by the PoseView program[42,43] on the PDB website (see Figure 7),

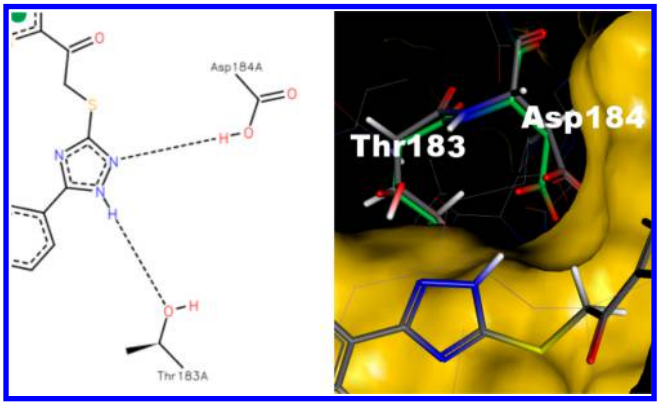

Figure 7. Alternative protonation states of PTV. On the left, the 1H form as proposed by PoseView[42,43] is shown (Adapted with permission from http://www.rcsb.org/pdb/home/home.do. Copyright 2015). For optimal hydrogen bonding, Asp184 has to adopt its protonated form acting as donor. On the right, the 3D visualization shows the interactions of the 2H form with Thr183 and the charged Asp184. This interaction pattern is more consistent with other PKA ligands. Additionally, the slight displacement of Asp184 resulting from the protein setup can be seen. In the original X-ray structure (green carbon atoms), the carboxyl group is closer to the ligand and forms a stronger hydrogen bond than in the energy-optimized conformation used as input in the docking (gray carbon atoms).

Thr183 and Asp184 act as hydrogen acceptor and donor, respectively. However, Asp184 has then to be in its protonated form. By moving the proton to the 2 position (2H form), Thr183 becomes the donor and Asp184 the acceptor, which is more consistent with many other X-ray structures of PKA, where Asp184 can unambiguously be identified as hydrogen bond acceptor or is even addressed by positive moieties of the ligands forming salt bridges. To take into account that the correct protonation of the triazole is very hard to predict even in solution and is probably binding site dependent, we generated all possible protonation states with our setup tool SPORES[44,45] and used them in the INPHARMA docking. Unfortunately, it was not possible to unambiguously identify the correct protonation state based on the docking scores. The main reason for this is the protein setup routine since the side chain of Asp184 is displaced from its position optimal for PTV binding during the minimization (see Figure 7). Even if a pose of the 2H form almost identical to the X-ray pose is found in some independent docking runs (right side of Figure 8), the hydrogen bonds to Thr183 and especially to Asp184 show very distorted geometries with long hydrogen-acceptor distances and contribute only little to the score. In other runs, better scored poses are generated (see e.g. left side of Figure 8), where the triazole forms hydrogen bonds to completely other parts of the binding site. Additionally, poses of the 4H form with Thr183 acting as acceptor (flipped ring) or donor (correct ring orientation) show very comparable or even better scores. We expect that such problems can be circumvented with the improved PLANTS version currently under development taking side chain flexibility into account.

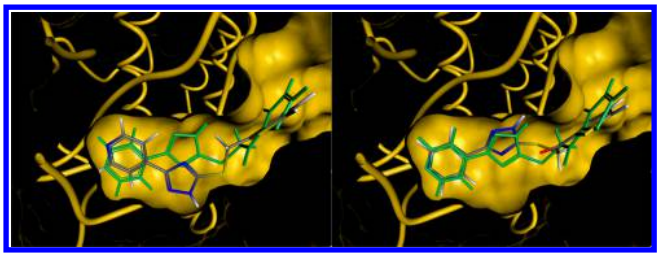

Figure 8. Poses of PTV resulting from docking runs including INPHARMA and trNOE data in combination with ligand M77 (runs 3 and 6; see the Supporting Information for more details). Due to modified side-chain orientations resulting from the protein setup procedure, the hydrogen bond to the hinge region and to Asp184 cannot be formed at the same time (right) resulting in a less favorable score and a higher scored false positive (left).

LL2: Ambiguous Ligand Pose. For ligand LL2, two poses with almost the same score are obtained in all docking runs, ChemPLP-only and INPHARMA, where the first one corresponds to the X-ray structure (at least with respect to the position of the thiazol-2-amine part, RMSD between 1.5 and 3.0 Å). The second one is obtained by a 180° rotation with an interchange of the two ring systems (RMSD above 5 Å). Depending on the conformation of the protein, one of these poses is ranked best, leading to a putative success or failure of the docking. In particular, the position of the side chain of Asp184 has a major impact. If the carboxyl group is oriented toward the ligand like in 3DNE, the amino group of the ligand can form a hydrogen bond with this residue, favoring the incorrect pose. If this side chain is treated as flexible in a ChemPLP-only docking, the incorrect pose is the highest-ranked one even using the native protein structure 3DND (data not shown). To understand this behavior as well as the large deviation of the phenyl ring even in the correct pose, we had a closer look at the crystal structures 3DND and 3DNE. While the ligand LL1 perfectly sits inside a cloud of high electron density in 3DNE, the pose of LL2 in 3DND is only partly covered by electron density (see Figure 9). Especially the phenyl ring, which shows many different orientations in the docking poses even if the thiazol-amine part is correctly placed, is badly defined. This indicates a high flexibility of the ligand and/or a very weak binding.[49] This flexibility has to be taken into account when evaluating INPHARMA with X-ray structures. On one hand, since the thiazol-amine moiety is

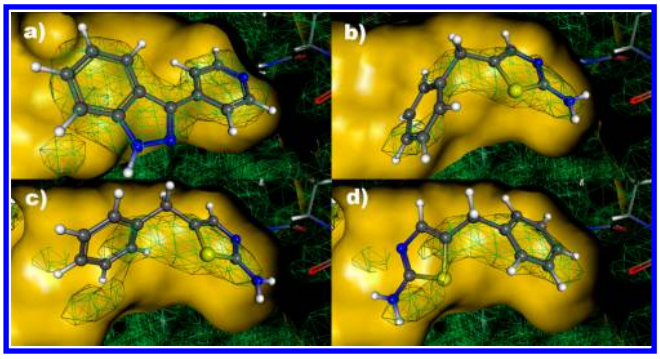

Figure 9. Electron density (green triangle mesh) of the ligands in the X-ray structure of PDB entries 3DNE (a) and 3DND (b). Additionally, two docking poses obtained from the ChemPLP-only dockings (c and d) are shown agreeing almost equally well with the experimental data as the X-ray pose.

DOI: 10.1021/acs.jcim.5b00235
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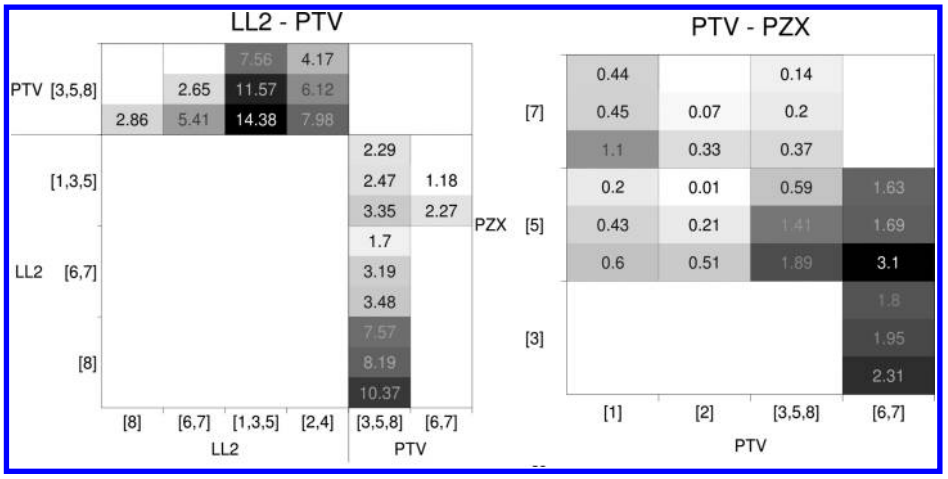

Figure 10. Volumes of the INPHARMA signals (adapted from ref 29) seen in the ligand combinations LL2−PTV (left) and PTV−PZX (right). Each dashed cell groups the integrals of three mixing times (300, 500, and 700 ms). (Adapted with permission from ref 29. Copyright 2013 American Chemical Society)

found more or less at the same site as in the X-ray structure in all docking poses with RMSDs between 1.5 and 3.0 Å, all these docking poses correctly reproduce the experimental structure. On the other hand, the 180° rotated pose fits almost as well into the electron density as shown in Figure 9 even if the highest electron density is not located at the sulfur atom. Thus, the experiment is not able to prove this pose wrong at least as a second possibility. The docking algorithm is also not able to distinguish between the two poses if the expected errors of the scoring function are taken into account. In many cases of unsuccessful dockings in standard test sets, the correct pose is placed on the second or third place with just a few scoring units difference to the best ranked pose. From this, we can deduce an error estimate for the score of 3−5 units being in the range of 2−10% of the total score (total scores of 50−150 units are seen in these standard sets). ChemPLP scores the 180° rotated pose (score = −67.36 units) only 2 units worse than the X-ray-like pose (score = −69.76 units). Even if no hydrogen bond to the hinge region of PKA is formed in the rotated pose, the better steric fit of the phenyl ring compared to the thiazol-amine part into the surface of the binding pocket leads to these very similar scores well within the error bar. In the case studied here, we can distinguish between the correct and incorrect pose because of the known X-ray structures. However, even if these structures were not available, two complex structures are delivered between which INPHARMA cannot distinguish. This is not too dramatic, since one starts from a situation where nothing is known about the complex structure and ends up with two possibilities. This might be helpful even as is to the synthetic chemist who wants to improve the affinity of the ligand. Also, as has been shown in ref 36, combination with trNOE and STD often resolves such ambiguities.

The impossibility to distinguish between two poses by INPHARMA has been seen in the previously published studies.[8,10,29] Very similar correlation coefficients between experimental and back-calculated INPHARMA signals are obtained for the two poses. There are many possible reasons, why these studies were able to identify the X-ray pose while the rotated pose is ranked highest in the docking presented here: (1) Different criteria are used for the evaluation of the poses (ChemPLP + INPHARMA vs INPHARMA only). (2) Due to the roughness of the INPHARMA score, slight deviations in the poses, which result from the different docking programs and scoring functions, can have a significant influence on the score. (3) Different protein conformations and side-chain orientations have a strong effect on the ChemPLP and INPHARMA score as discussed for the latter in the work of Stauch et al.[46] (4) Including additional information from other ligand pairs as done in the INPHARMA-STRING method helps in identifying the correct pose. Besides point 4, the changes in the scores result from minor perturbation in the molecular system and we strongly advise against basing conclusions on such small differences in the scores. Therefore, we conclude that the pose of ligand LL2 cannot be determined unambiguously based on docking with or without INPHARMA data using a single ligand pair. Additionally, multiple poses may exist for this small, fragment-like ligand as discussed above. This possibility is further supported by the similarity of the thiazol-amine moiety with the 1,2,4-triazol-3-yl-sulfanyl moiety of PTV regarding pharmacophoric features, which renders an attractive interaction with THR183 and ASP184 very likely. With all these uncertainties, we regard LL2 as an unsuitable example for method validation.

PTV−PZX and LL2−PTV: Insufficient and Ambiguous Experimental Data. The INPHARMA spectrum of the PTV−PZX pair shows only eight interpretable INPHARMA peaks (see Figure 10). Additionally, the integrals of these peaks are very small and of low variance, i.e. they all have almost the same size. Thus, they correspond to large distances between the corresponding nuclei, which can be fulfilled in very different relative orientations of the two ligands. Due to experimental errors, which are expected to be especially prominent for smaller peaks, and structural uncertainties (e.g., suboptimal side-chain orientations discussed above), complete agreement between experiment and back-calculation cannot be expected. It was theoretically proven[47,48] that the correlation coefficient of a perfect model depends on the ratio between experimental error and variance. Nevertheless, very high correlation coefficients, i.e very good INPHARMA scores, are seen in the docking experiment for a large number of wrong orientations, which all exceed the expected value considering the results from the just cited publications. This can only be explained by overfitting, i.e. the docking tries to optimize the poses in a way that they describe the stochastic errors of the experiment. Therefore, the INPHARMA score only introduces additional noise into the docking run. For LL2−PTV also very few

DOI: 10.1021/acs.jcim.5b00235
J. Chem. Inf. Model. 2015, 55, 1962−1972

INPHARMA peaks are observed (Figure 10). Even if the integrals are larger, very little information can be obtained from these, due to the ambiguous assignment of chemical shifts of PTV. Seven of the eight peaks are seen between nuclei of LL2 with the resonance assigned to the three atoms 3, 5, and 8 of PTV. As seen in Figure 1, atom 3 is on the opposite side of the molecule than atom 5 and 8. Thus, independent of the actual position of PTV, these constraints can be fulfilled as long as PTV occupies, at least partly, the same region in the binding site as LL2. In summary, the INPHARMA data for these two pairs cannot be used quantitatively in the current implementation of INPHARMA docking. Qualitatively, regarding the signal-to-noise and the number of INPHARMA NOEs, this ligand pairs did not reach the number or quality of NOEs seen for other combinations. More work has to be invested to develop criteria evaluating the quality of the INPHARMA data and the information content in the spectra. If then one or several of these criteria falls below a specific limit, which has to be defined using additional test systems, the INPHARMA data should be interpreted with care. Nevertheless, in combination with other experiments as done e.g. in the INPHARMA-STRING method,[29] the data can still be beneficial.

Improved Setup. As shown with the examples above, the combined usage of INPHARMA and trNOE data is able to remove false positive poses generated by the ChemPLP scoring function. Additionally, ligand preparation especially regarding protonation states was identified to be important for aromatic rings containing multiple nitrogen atoms. Therefore, the docking runs for all pairs were redone using these setting and the optimal protomers. The results are given in form of a heat map in Figure 11 (scores, RMSD values, and visualization of

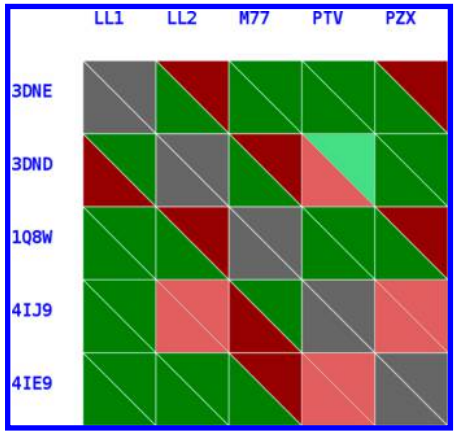

Figure 11. Heat map of the successes for the INPHARMA docking experiments using the improved setup. The lower and upper triangle in each square corresponds to the native and the cross-docked ligand, respectively. Pairs with insufficient experimental data are shown in lighter colors.

best poses are given in the Supporting Information). LL1, M77, and PTV are predicted correct in almost all combinations. The only exception is the M77/PTV pair. In the latter, the suboptimal protein conformation worsens the ChemPLP but also the INPHARMA score so strongly that false positives can outrank the correct pose. LL2 adopts in many combinations still the 180° rotated pose. As discussed above, it is not absolutely clear that this pose is not, at least partly, adopted by the ligand due to missing and ambiguous electron density of the ligand in the crystal. Finally, the PZX ligand was predicted correctly in three combinations (LL1/PZX in protein structure 4IE9 and LL2/PZX in both protein structures). Thus, the scoring problems of ChemPLP can be removed for this ligand. In these successful dockings, the 1H protonation was used (N^1 is protonated in contrast of the original N^2 protonation, labeling according to Figure 1). For LL1 and for PTV, the original protonation and the 2H form (following the discussion above) is adopted in the optimized setup, respectively.

As just mentioned, the combined score built from ChemPLP, INPHARMA, and trNOE shows a clear improvement of the results for ligand PZX compared to the ChemPLP-only runs. In combination with LL1, poses were generated with both ligands well inside the binding site of protein structure 4EI9 and RMSD values are below 2 Å. The visualization of the interaction pattern of PZX by the PoseView program[42,43] on the PDB Web site shows that, besides the pyridine ring binding to the hinge region, important interactions are formed by the ester group and the pyrazole ring to residues Thr183, Asp184, and Lys72 (see Figure 12). These are only seen weakly in the

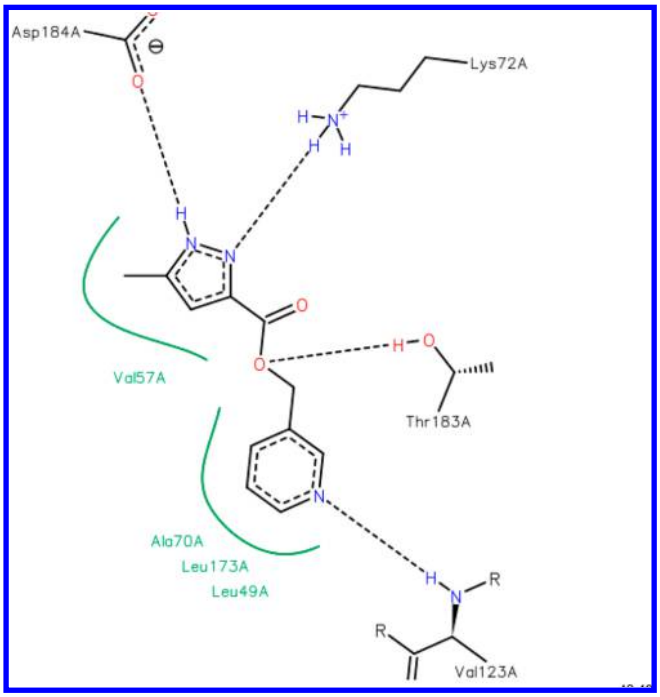

Figure 12. Interaction pattern of ligand PZX in the binding site of PKA visualized by PoseView[42,43] (Adapted with permission from http://www.rcsb.org/pdb/home/home.do. Copyright 2015). Besides addressing the hinge region, the ligand forms hydrogen bonds to Thr183, Lys72, and Asp184 in the X-ray structure. The latter two cannot be preserved after the energy optimization of the free protein during the setup procedure.

structure used as input for the docking. Asp184 and Lys72 are very flexible and adapt to specific ligands. As described in the Materials and Methods section, the reference structures were taken from the work of Skjaerven et al.[29] The protein setup performed there applied a homology modeling to standardize atomic nomenclature including energy minimization. Even when the starting structures were the protein−ligand complexes, the ligands were not included in the setup procedure and therefore, side chains adopt conformations optimal in the ligand-free protein but not the complex. Without the possibility to recover the optimal geometric arrangement especially of hydrogen bonds (only when side-chain flexibility is

DOI: 10.1021/acs.jcim.5b00235
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enabled in the docking, these side chains are able to flip back to the optimal ligand-bound conformation), the score of the correct pose worsen moving it to lower ranks in the ChemPLP-only dockings. Adding the INPHARMA information removes false positives since the relative orientations of the two ligands seen in these would result in intermolecular NOE signals inconsistent with the experiment. This leads to partly successful dockings for PZX (the pair LL1−PZX is predicted correctly in the 4IE9 protein structure) compared to the complete failure of the ChemPLP-only dockings. Thus, INPHARMA helps to reduce the influence of uncertainties in the protein structures which are known to reduce the success rates of cross-dockings (docking into a protein structure, which was solved with a different bound ligand) and especially of dockings based on homology models.

However, some ambiguities still remain. The two best scored poses from the independent docking runs are show in Figure 13. The orientation of LL1 is the same in both and also the

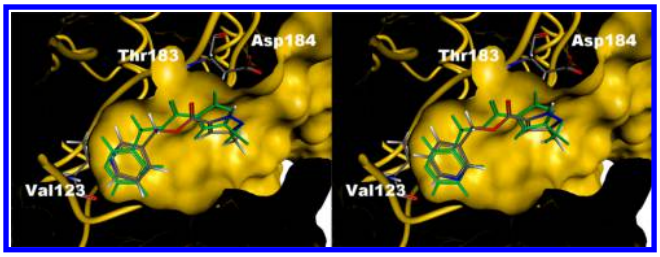

Figure 13. Poses of PZX resulting from docking runs including INPHARMA data in combination with ligand LL1 (run 12 and 15, see the Supporting Information for more details). Due to modified side-chain orientations resulting from the protein setup procedure, the hydrogen bond to the hinge region cannot be formed in the optimized poses and the score is almost independent of the orientation of the pyridine ring. Lys72 is not marked since it lies in front of the ligand in this orientation.

placement of the ester group and the pyrazole ring of PZX is almost identical. Only the pyridine ring is flipped in the second pose getting almost the same score as the first. This is interesting since the pyridine is, as already mentioned above, known to form the interactions with the hinge region (Val123). Better scores are obtained, when the hydrogen-bonding interaction of the ester group to Thr183 is optimized by the docking approach resulting in the slight displacement of this group compared to the reference structure. At the same time, the steric interactions are strengthened and, even more importantly, the agreement of the experimental and back-calculated INPHARMA spectrum is improved, enhancing the INPHARMA score by almost 20 units (X-ray pose: total score = −66.08 units, INPHARMA score = 39.58 units, optimized poses: total score = −91.47 units, INPHARMA score = 21.33 units). This improvement is, however, only possible at the expense of a weakening of the binding to the hinge region. The hydrogen bond to the hinge region is accordingly not formed in neither of the docking poses of PZX. Once more, even more accurate results should therefore be obtainable if Asp184, Lys27, and perhaps Thr183 are treated as flexible. In this way, hydrogen bonds to all these groups simultaneously to the hinge-region hydrogen bond should be able to form. As mentioned above, side-chain flexibility is implemented in PLANTS but it is not applicable to INPHARMA docking at the moment due to conflicting demands of the two ligands. We are working on an improved implementation, where independent protein structures are used for each ligand. This will allow for the simultaneous adaptation of the side chain or even the backbone (by providing an ensemble of structures) to each ligand. Most likely, other ligand pairs like LL1-PTV and M77-PTV discussed above will also benefit from side-chain flexibility and the procedure should probably generally be applied. PKA is very demanding on the side chain placement as can be seen also from the cross-docking experiments described above. Even in cases where the pose is predicted correctly, important interactions are missed due to the large differences in the side-chain orientations in the X-ray structures. Consequently, much lower scores are obtained for the cross-docking than for the redocking runs.

CONCLUSION

Here we have presented the first approach to combine a standard scoring function like ChemPLP with scores evaluating the agreement between experimental and back-calculated spectroscopic data to increase the reliability of docking procedures. Even if this approach is able to remove false positives, which are highly ranked by the standard scoring function but which do not agree with the experimental INPHARMA and transfer NOE data, we were not able to predict all ligands correctly based on only one INPHARMA measurement. Different reasons for these failures could be identified and partially removed. One very important factor not only for INPHARMA but for all docking approaches is the preparation of the complex. The nitrogen-rich aromatic rings of the ligands used here have many possible protonation states and the correct one is probably binding site dependent. Therefore, all these protonation states should be used in the docking and the correct one can then be identified by numerically comparing scores or by visual inspection of the interactions.

Additionally, due to the rearrangement of side-chains taking place during the protein setup, only weak hydrogen bonds can be formed between protein and some ligands or these hydrogen bonds are even broken completely. This results in a substantial worsening of the score of the correct pose and the increased occurrence of false positives. Using side-chain flexibility especially for the residues Asp184 and Lys72, extremely changing their orientation to specifically address the ligands, should further improve the results. Besides these easy visible destruction of interactions influencing the ChemPLP score, the INPHARMA score is also strongly dependent on the side-chain orientations as demonstrated by Stauch et al.[46] We are working on an improved PLANTS version, in which the side chains can adapt to each ligand separately and even different, optimal backbone conformations can be chosen. Unfortunately, adding these additional degrees of freedom will aggravate a problem already seen in the calculations performed here. Even with the inclusion of trNOE data, which improved the convergence of the search algorithm, we saw sampling problems especially for larger, more flexible ligands. The final scores in independent runs could differ by 10−20 units and optimal scores could be sometimes only obtained in a couple or even one of the 6−10 individual run (see the Supporting Information). Standard PLANTS runs even with much larger ligands converge within a few units. Trying to avoid these sampling problems by more intense search settings (50 ants, σ = 1.5) was not successful. Thus, only the large number of independent runs provides some guarantee that the important local minima are sampled and the global one is found and minimizes the influence of

DOI: 10.1021/acs.jcim.5b00235
J. Chem. Inf. Model. 2015, 55, 1962−1972

sampling on the results. For all examples shown here including the failures, poses very close to the X-ray structures were generated in the docking runs and ranked first in some of the individual runs but, in the case of a failure not the best one, or on lower ranks. Thus, all failures can be considered as scoring errors and not sampling errors, where the second describe cases in which the correct pose in not found because it is never generated by the sampling algorithm (false negatives). Nevertheless, improving the convergence properties would improve the confidence in the results and lower the computational demand. Interestingly, the increased complexity of the optimization problem is not introduced by the INPHARMA score but by the simultaneously optimization of the two ligands, which was proven by ChemPLP-only runs using a pair of noninteracting ligands (data not shown). The challenge seems to be that two optimization problems have to be solved at the same time, which are only weakly coupled by the INPHARMA score or not at all in the ChemPLP-only runs. We are experimenting with different update schemes for the pheromones to overcome this problem.

Stauch et al.[46] also discussed that besides the average conformation of the protein also protein dynamics has a significant influence on the INPHARMA signals and their back-calculation. By including internal motion described by order parameters derived from molecular dynamics, they were able to obtain a better representation of the experimental data. Including these effects in the docking is only possible with an extreme increase in computational demand, since the order parameters have (1) to be determined and (2) to be included in the evaluation of every pose generated by the docking algorithm. Additionally, criteria beyond Pearson correlation coefficients to evaluate the agreement between experiment and back-calculation have to be developed for INPHARMA including dynamics.

Remaining failures not associated with complex preparation can be attributed to insufficient experimental data (LL2−PTV and PTV−PZX) and ambiguous ligand poses in the X-ray structures, from which the first problem can easily be circumvented by combining different experiments like it is done in INPHARMA-STRING[29] or STI.[30] Since the scores for the false positives rarely was much better than for the correct positives, they can be identified in most cases just by observing several complex structures instead of 1.

In summary, the study presented here was designed to generate additional know-how about how to use the information on a single INPHARMA experiment most effectively. Results show the potential of an INPHARMA-guided docking approach but also the challenges, which have to be overcome regarding scoring and sampling problems. Work is ongoing to incorporate the generated know-how into an advanced method including side-chain flexibility and new pheromone update scheme, which will to be tested on a larger test set (when this becomes available) hopefully leading to a general INPHARMA-ChemPLP scoring function and a standard protocol for its use in docking. This will then be combined with approaches taking the information on multiple INPHARMA experiments into account like the INPHARMA-STRING method[29] or STI potentially combined with multiplexing.[30] In this way, more reliable results should be generated profiting from the better poses for a specific pair and by consensus poses over multiple pairs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jcim.5b00235.

Representative INPHARMA spectra for the ligand pairs LL1−M77 and LL1−PTV. Docking scores and RMSD values of the highest ranked poses of each independent docking run as well as figures showing the overall best poses (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Bernhard Rupp for his helpful discussion on the X-ray structures of the PKA−LL1 and PKA−LL2 complexes. This work was supported by the German Federal Ministry of Education and Research (BMBF, Grant No. 1615870D) and the Zukunftskolleg of the Universität Konstanz. C.G. was supported by the Max Planck Society.

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DOI: 10.1021/acs.jcim.5b00235
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