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Performance of The MM/GBSA Scoring Using a Binding Site Hydrogen Bond Network-Based Frame Selection: The Protein Kinase Case

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ABSTRACT

A conformational selection method, based on hydrogen bond (Hbond) network analysis, has been designed in order to rationalize the configurations sampled from molecular dynamics (MD), which are commonly used in the estimation of relative binding free energy of ligands to macromolecules through MM/GBSA or MM/PBSA methods. The approach makes use of protein-ligand complexes obtained from X-ray crystallographic data, as well as from molecular docking calculations. The combination of several computational approaches, like long MD simulations on protein-ligand complexes, Hbond network-based selection by scripting techniques and finally MM/GBSA; provides better statistical correlations against experimental binding data than previously similar reported studies. The approach has been successfully applied in the ranking of several protein kinase inhibitors (CDK2, Aurora A and p38), which present both diverse and related chemical structures.

INTRODUCTION

One of the main challenges that face a chemist (medicinal, organic, inorganic, etc.) is a deeper understanding of the forces that are involved and govern a chemical reaction or the intermolecular interactions that arise when two molecules are close enough to form a stable molecular complex (protein – protein, protein – ligand, carrier – drug, etc.). Computational methods currently available are of key importance in understanding complex processes taking place at the molecular level. Several different computational approaches exist specifically to assess the protein – ligand interactions. The spectrum of methods is broad, both in complexity and demanding computational time, where cheaper computational methods like molecular docking attempt only to predict the structure (or structures) of inter-molecular complexes formed between two or more molecules; therefore it has been widely used to suggest the binding modes of protein inhibitors.[1,2] Most of the docking algorithms are able to generate a large number of possible structures, so they also require a means to score each structure to identify those of most interest. The "docking problem" is thus concerned with the generation and evaluation of plausible structures of intermolecular complexes.[3,4] The main disadvantage of docking methods is their scoring function, because it may become insufficient to adequately represent all binding forces and to rank a set of inhibitors properly. Some improvements have been made in this regard and new algorithms are appearing that seem promising to face this challenge.[5,6]

Next, in the scale of complexity, are the quantitative structure-activity relationship (QSAR) methods. QSAR relates structural properties of the molecules to its activity by a mathematical model. The term "quantitative structure-property relationship" (QSPR) is also used, particularly when some property other than biological activity is concerned. In drug design, QSAR methods have often been used to consider qualities beyond in vitro potency. The first use of QSARs to

rationalize biological activity is usually attributed to Corvin H. Hansch. He developed equations which related biological activity to a molecule's electronic characteristics and hydrophobicity.[7] QSAR methods fail in the sense they are not predictive at all and, in order to build the QSAR equation, one needs to include several non-correlated variables into that equation to get the final model. Some of these variables (or descriptors) may not have chemical meaning and render useless the method as a reliable structural interpretative tool. In the middle of the computational methods spectrum is the molecular dynamics (MD) simulation. This approach is very powerful for the molecular description of very large systems like DNA – drug complexes[8,9], polymer structures[10,11], membrane proteins[12,13], protein – ligand complexes[1,14], etc. The simplicity of the employed potential energy function of this method makes it suitably to run very long simulations, even in the millisecond time scale[15, 16], and then allowing to extract valuable structural information and energy data from the resulting trajectories. Other more refined molecular dynamics approaches like MD-FEP allowed to calculate "absolute" protein – ligand binding free energies.[17,18] A general methodology for calculating the equilibrium binding constant of flexible ligand to a protein receptor was formulated by Woo H-J *et al*,[19] on the basis of potentials of mean force (PMF). The mentioned approach avoids the need to decouple the ligand from its surroundings (bulk solvent and receptor protein), as it is traditionally performed in the double-decoupling scheme, but those approaches are computationally expensive and are not applicable to any kind of compounds due to the limitations of molecular force fields. That force field's drawback, in the treatment of organic-inorganic drug like compounds, can be solved by means of hybrid calculation methods like QM/MM and/or ONIOM.[20,21] On the other hand, the "relative" binding free energy may be estimated on the basis of a continuum solvent approximation like the Molecular Mechanics/Generalized Born and Surface Area (MM/GB-SA)

method [22,23]. A popular procedure of this approach relies on a mixed scheme combining configurations sampled from MD simulations with explicit solvent, together with free energy estimators based on an implicit continuum solvent model obtained from MM/GBSA calculations.[24] The main advantage of this protocol is the use of an ensemble of structures (snapshots or frames) accounting for the conformational flexibility of the protein-ligand complex.[25] Furthermore, through the application of the conformational sampling to generate ensemble averages, one can also simulate better the protein-ligand reciprocal adaptation, a phenomenon commonly referred to as induced-fit.[26] Recently, some studies have been performed in order to assess the impact of several parameters (force field, ligand charge models, length of molecular dynamics simulations, among others) on the performance of MM-GBSA and MM-PBSA methods.[27–29] It is worth also to mention that, due to popularity among computational chemistry community and to the relative good accuracy of this binding free energy calculation approach, some automated programs has been developed by Gohlke *et al*.[30, 31] in order to allow a more straightforward application of this methodology.

Concerning the induce-fit concept, some of the previous research work relied on rigid protein-ligand docking structures; and that structural effect, which is known to be important in the binding of small molecules to proteins and other macromolecular targets, was mostly ignored. For instance, Du *et al*.[2] applied multiple docking strategies and Prime/MM-GBSA calculations to predict the binding modes and free energies of a series of benzoisoquinolinones as Chk1 inhibitors. The authors found that reliable docking results were obtained using induced-fit docking and quantum mechanics/molecular mechanics (QM/MM) docking, which showed superior performance on both ligand binding pose and docking score accuracy to the rigid-receptor docking. Then, the Prime/MM–GBSA method based was applied to the docking

complexes to predict the binding-free energy. Among other targets, protein kinases are particular flexible proteins, so that induce-fit effects should be considered in the structure-based design of kinase inhibitors.[32]

In this work we present a novel interaction-based selection approach of representative structural configurations obtained from long MD simulations of several protein kinase-inhibitors complexes, which were then subject to MM/GBSA calculations in order to estimate the corresponding relative free binding energies. Geometrical parameters (distance and angle) for each Hbond were derived from equilibrium geometries exhibited in MD trajectories that were weighted in order to score and select those protein-ligand configurations that best represent the overall intermolecular interaction behavior. Three protein kinase systems were considered: p38, Aurora A and CDK2 (Table 1). The inhibitors for p38 and Aurora A are congeneric series previously reported[33,34] and their protein-ligand complexes were obtained from docking experiments. The inhibitors for CDK2 are non-congeneric series making the scoring through MM/GB-SA more challenging. However, in this case the protein-ligand complexes were obtained from crystallographic data available in the Protein Data Bank (PDB)[35]. The proposed computational protocol seems to capture a relevant feature within binding site in these protein kinase systems, the so-called "Hbond network", present at the protein hinge region, which most inhibitors reported so far establish interactions with.[36, 37, 38]

Please Insert Table 1 around here

METHODS

Data Sets and System Setup. A CDK2 protein-ligand data set with X-ray crystal structures previously collected and reported by Dobeš *et al*.[39] were used as the non-congeneric series. p38 (PDB ID: 2bak) and Aurora A (PDB ID: 2c6e) kinase data sets previously collected and reported by Lyne *et al*.[40] were used as the congeneric series. The *in vitro* biological activity data reported as K_i or IC_{50} values in the above-mentioned literature for inhibition produced by diverse and related chemical derivatives on different protein kinase enzymatic systems were used. For modeling purposes, IC_{50} values were converted into binding free energy (ΔG) values. Biological data for the CDK2 set was used as natural logarithm of K_i values (pK_i). All X-ray crystal structures were prepared, refined and completed (when needed) with the Protein Wizard Preparation module available in Maestro[41] visualization software. Prediction of missing loops was made with Prime[42] module from Schrödinger Suite. All compounds of the congeneric series of p38 and Aurora A were prepared with the software LigPrep (LigPrep, version 2.5, Schrödinger, LLC, New York, NY, 2011), while the protonation states were predicted via the Epik (Epik, version 2.2, Schrödinger, LLC, New York, NY, 2011) program.[43] The analysis was performed at biological conditions, i.e., using water as a solvent and pH 7.0. All obtained tautomers were further used in docking experiments. In Table 1 are shown the reference crystal structures for each series, the number of compounds used in the series and the resolution of each crystallographic structure.

Molecular Docking Simulations. Docking experiments were performed, for p38 and Aurora A inhibitors starting from reference X-ray crystal structures (PDB id codes: 2bak and 2c6e,

respectively), using Glide in standard precision (SP) mode[44–46]. Glide docking uses a series of hierarchical filters to find the best possible ligand binding locations in a previously built receptor grid space. The filters include a systematic search approach, which samples the positional, conformational, and orientational space of the ligand before evaluating the energy interactions between the ligand and the protein. A grid box of 30Å×30Å×30Å was first centered on the reference ligand co-crystallized with each targeted protein and default docking parameters were used. The docking hierarchy begins with the systematic conformational expansion of the ligand followed by placement in the receptor site. Then a minimization of the ligand in the field of the receptor is carried out using the OPLS-AA[47] force field with a distance-dependent dielectric of 2.0. Afterwards, the lowest energy poses are subjected to a Monte Carlo (MC) procedure that samples the nearby torsional minima. The best pose for a given ligand is determined by the Emodel score, while different compounds are ranked using GlideScore, a modified version of the ChemScore function described by Eldridge *et al*.[48] that include terms for buried polar groups and steric clashes. The docking poses for each ligand were analyzed by examining their relative total energy score. The more energetically favorable conformation was selected as the best pose for further computational experiments. In Figure 1 is shown an overlay of the molecular docking conformations obtained for all inhibitors within the binding site of p38 and AuroraA kinases. Those conformations were used as starting point for subsequent long molecular dynamics simulations.

Molecular Dynamic Simulations. Molecular dynamics of all compounds within CDK2, p38 and Aurora A binding sites were studied using the OPLS – AA force field in explicit solvent, employing the SPC water model (OPLS-AA/SPC) [49], with the Desmond [50, 51] package for MD

simulations. The initial coordinates for MD calculations were taken from reported X-ray complexes (CDK2) or from the performed docking experiments (p38 and Aurora A). For solvation of the systems an orthorhombic water box was used, which dimensions ensures a buffer distance of approximately 10Å between each box side and the protein atoms. This guarantees the whole surfaces of the complexes to be covered when the SPC water molecules were added. The protein ligand systems were neutralized by the addition of Chloride counter ions. After the solvent environment construction, about 34000 atoms composed each protein ligand system. Before equilibration and long production MD simulations, the systems were minimized and pre-equilibrated using the default relaxation routine implemented in Desmond. For this, the program ran six steps composed of minimizations and short (12 and 24 ps) molecular dynamics simulations to relax the model system before performing the final long simulations. After that, a first 2 ns short equilibration MD simulation was performed on each complex system that was followed by a 10 ns long production MD simulation. The OPLS-2005[49] force field was used, along with the module MacroModel[52] to provide and check the necessary force field parameters for the ligands. When MacroModel performs an energy calculation, the program checks the quality of each parameter in use. Use of low-quality parameters, especially torsional ones, may result in inaccurate conformational energy differences and geometries. All, bond, angle, torsional and improper angles parameters were listed as high- and medium-quality force field parameters for all the studied ligands.

During MD simulations the equations of motion were integrated with a 2 fs time step in the NPT ensemble, where temperature (300K) and pressure (1atm) were maintained using Nosé-Hoover thermostat and Martyna-Tobias-Klein (MTK) barostat methods, respectively. The SHAKE

algorithm was applied to all hydrogen atoms; the van der Waals cutoff was set to 9.0Å. Long-range electrostatic forces were taken into account by means of the particle-mesh Ewald (PME) approach. van der Waals (vdW) and short range electrostatic interactions were smoothly truncated at 9.0Å and the equations of motion were integrated using multistep RESPA integrator with 2.0 fs inner time step for bonded or near non-bonded interactions and 6.0 fs for far non-bonded interactions. Periodic boundary conditions and restraints were applied to backbone (0.5 kcal/mol) and ions (3 kcal/mol) in all cases to ensure structural stability. Data were collected every 3 ps during the MD runs. Visualization of protein-ligand complexes and MD trajectory analysis were carried out with Maestro. Root mean square deviation (RMSD) analysis for first 2 ns of MD was performed for all protein-ligand systems studied before starting long MD simulations. These analyses showed good structural stability for all equilibration MD simulations recorded. In Figure 2 are shown the plots of RMSD against simulation time for the most potent compounds in each series studied (See Figure 1SI in Supplementary Information section for RMSD of the remaining ligands).

Binding Site Hydrogen Bond Network Analysis. It is well known that most of protein kinase inhibitors establish several hydrogen bonds with residues located at the hinge region, which are important for binding stability.[36–38] In order to efficiently address the analysis of these interactions, a recursive algorithm that progressively evaluates the dynamics of the Hbond network, between ligands and protein kinase-binding site, along the MD trajectories was developed. The aim was to systematically identify those interactions that may be significant for the binding behavior. The algorithm searches for the standard Hbond geometry (see Scheme 1), which is defined by distances and certain angles between near hydrogen bond acceptor (HBA)

and hydrogen bond donor (HBD) atoms.[53] The algorithm was also coded to allow the study of Hbond interactions mediated by one or two water molecules, since the protein kinase binding site is accessible to the solvent and large enough to allow the access of water molecules together with the compound into the cavity. Search parameters can be tuned to match alternative Hbond geometries such as C – H weak hydrogen bonds[53], as well as set Hbond equilibrium length and threshold time (minimum time span to consider it as a significant interaction; by default is 500ps).

Please Insert Scheme 1 around here

Conformational Selection Analysis Based on HBond Network. Once the Hbond interactions' relative occurrence had been determined, two conformational selection methods were applied in order to select the protein ligand structures (frames) from MD trajectories, which then were subject to binding free energy calculations through MM/GBSA method.

First, a random selection was performed while enforcing a well-spread sampling from a given MD protein-ligand conformational population by splitting it into several equally-sized samples so then randomly choose one structure from each of them. That could ensure a decent and more representative selection of structures while still being completely random.

Second, an interaction-based selection method was implemented, that allowed us to select those protein-ligand conformations that presented optimal Hbond interaction geometries according to calculated geometrical equilibrium values. To do so, measurements of picked Hbond interactions were computed through the entire simulation time, which were defined in an Interaction

Specification Format (ISF) file (See Supplementary Information Section). Based on the collected information, every MD trajectory frame was rated by calculating the deviation from the reference Hbond geometry by a weighted scoring function defined as:

$$S_f = \sum_{m}^{k} i_m \times \left|1 - \frac{m_f}{m_0}\right|, \qquad \sum_{m}^{k} i = 1 \tag{1}$$

where m_f represents a single geometrical measurement (i.e. distance, angle and/or dihedral) between near HBD and HBA atoms in the frame f of the MD trajectory, while m_0 represents the reference value; both parameters were also included in the ISF file. Additionally, it is possible to give a statistical weight to those interactions considered to be crucial in the ligand binding by assigning them an importance factor i_m, while ensuring that their sum is normalized to 1. It is important to note that, in general, geometrical Hbond reference values are automatically estimated by calculating their statistical mode along the MD trajectory, which guarantees representative values for the overall binding behavior sampled by MD the simulation; however one can set reference values manually to explore particular interaction geometries of interest. Finally, all MD frames were ranked according to their score, where those with lowest value were selected as the representative structures to be used in MM/GBSA binding free energy calculations.

MM/GBSA Free Binding Energy Calculations. Computational methods that combine molecular mechanics energy and implicit solvation models have been widely exploited in free energy calculations, therefore their applicability and performance has been addressed in several protein-ligands systems in recent years[54–57]. Besides exhibiting a good accuracy and affordable

computational costs, they allow for rigorous free energy decomposition into contributions originating from different types of interactions. In this study, protein kinase-ligand free binding energies were calculated as the difference between the energy of the bound complex and the energy of the unbound target and inhibitor compound, as it is shown in Equation 2:

$$E_{binding} = E_{complex} - E_{protein} - E_{ligand} \quad (2)$$

Specifically, after calculating the energy of the protein-ligand complex, the ligand and the protein were separated, and their energies were computed using OPLS-AA force field with generalized Born implicit solvent model, in order to calculate the averaged binding free energy (ΔG) according to following equation:

$$\Delta G_{binding} = \Delta E_{MM} + \Delta G_{PB/GB} + \Delta G_{SA} - T\Delta S \quad (3)$$

where ΔE_{MM} includes $\Delta E_{internal}$ (bond, angle, and dihedral energies), $\Delta E_{electrostatic}$ (electrostatic), and ΔE_{vdw} (van der Waals) energies; $\Delta G_{PB/GB}$ is the electrostatic solvation energy (polar contribution), and ΔG_{SA} is the nonelectrostatic solvation component (nonpolar contribution). The polar contribution is calculated using either the Generalized Born (GB) or Poisson Boltzmann (PB) model, while the nonpolar energy is estimated by solvent accessible surface area (SASA).[58] Note that the implicit solvent model estimates solvation free energies, and thus, this energy implicitly includes entropies associated with solvent. The conformational entropy change -TΔS is usually computed by normal-mode analysis on a set of conformational snapshots taken from MD simulations.[56]

The MM/GBSA approach was used as implemented in Prime[42] module from Schrödinger Suite and using default settings. Protein kinase-ligand frames were extracted from long MD simulations (10 ns) according to the selection methods described before. Six subsets of frames with different populations (10, 20, 50, 100, 150 and 200 frames) were selected in order to compare the performance of both selection methods in predicting the averaged relative binding free energies of the protein kinase systems selected for this study. During the simulation procedure, the ligand strain energy was also considered. No entropy contribution was computed for the studied protein-ligand systems to save computational time given the comparative purposes of the study. Finally, the computed free binding energies were plotted against experimental p$\mathbf{K}_i$ values, in CDK2 series, and against experimental ΔG values for p38 and AuroraA series obtained by the relation:

$$\Delta G = -RT \ln IC_{50}$$

where IC_{50} is the experimental biological activity. The degree of statistical correlation between the two (computed vs. experimental) ΔG values, using the correlation coefficient $\mathbf{R^2}$, was reported as well.

RESULTS AND DISCUSSION

In the present work we introduced a relative binding free energy calculation protocol, based on MD and MM/GBSA methods, that incorporates a novel interaction-based structure selection approach, which was applied to three series of diverse and chemically related protein kinase inhibitors. We have compared two selection methods to assess the performance of this new technique: the typical random selection method (using several frames randomly or time-dependently selected) and our interaction-based method, which was developed taking into account the typical geometrical parameters for hydrogen bonding interaction. To do so, the protein kinase-ligand complexes were obtained from X-ray structural data collected previously from PDB[39, 59] (in case of CDK2) or from molecular docking experiments (p38 and Aurora A) performed on available protein-derivative X-ray crystal structures deposited in PDB.[40] The corresponding PDB codes for X-ray crystal structures used in this study are reported in Table 1.

In all cases, the nature of the chemical structure of the considered inhibitors differs; for instance, CDK2 inhibitors are a non-congeneric series that could be problematic to study since it has been reported that this kind of molecular sets make the MM/GBSA scoring more challenging.[55] On the other hand, the p38 and Aurora A inhibitors are congeneric series, whose relative binding affinity is supposed to be more straightforwardly estimated by empirical binding free energy methods such as the employed here. According to previous studies, the MM/GBSA method in conjunction with molecular docking, provides a protocol suitable to rank relative binding affinities within congeneric series. For instance Lyne *et al.*[40] used an MM/GB-SA scoring protocol to correctly rank a number of congeneric kinase inhibitors, which were previously bounded to protein targets

through molecular docking. They found that such computational protocol of using molecular docking (with Glide [43]) for pose generation and MM/GB-SA method for rescoring appeared promising for the application to structured-based lead optimization of chemical series for inhibition of protein kinases. More recently, Kalyanaraman *et al*.[60] reported the "Prime-ligand" method for ranking ligands in a congeneric series. The method employed a single scoring function, the OPLS-AA/GBSA molecular mechanics/implicit solvent model, for all stages of sampling and scoring. They evaluated the method using 12 test sets of congeneric series (including those inhibitors of kinases reported by Lyne *et al*.[40], except Jnk-3 kinase because there was no crystal structure available in the PDB database at the time[35]) for which experimental binding data were available in the literature, as well as the structure of one member of the series bound to the protein. Despite the fact that their results for congeneric series were promising, and better than previous ones for protein kinases (p38 and Aurora A), they stated that allowing the receptor flexibility in the reported test cases reduced the correlations between the computed and measured binding affinities, being a quite dramatically disagreement in some cases. Thus, at least in their experience, the ability to rank/order compounds within congeneric series required use of a rigid receptor, presumably due (in part) to a greater cancellation of error.

Moreover, it is also known that results from free binding energy calculation using methods such MM-GBSA can be heavily influenced by the applied simulation protocols, specifically the sampling strategy of generating and selecting the snapshots[55].

These previous computational data on binding free energy estimation on protein-kinase systems, pave the way to perform further computational simulations in order to check three relevant points related with the application of the MM/GBSA approach in those systems: (1) the capability of

the approach to rank congeneric as well as non-congeneric series of protein kinase inhibitors, (2) the impact of including the protein flexibility through MD simulations, and (3) the selection analysis method employed in choosing the MD snapshots or frames. In the present research work, the abovementioned points were tackled using some protein kinases-ligand complexes as test systems.

Molecular Docking Simulations

In Figure 1 are shown the alignments of p38 (13 compounds) and AuroraA (13 compounds) inhibitor structures, obtained from molecular docking experiments, within their respective binding sites. As it can be seen, most of the p38 kinase inhibitors could establish Hbond interactions with the backbone amide NH of residue Met109, which belongs to the protein hinge region. There were further Hbonds between the amide NH of some compounds and the carboxylate side chain of residue Glu71, and between the amide carbonyl and the backbone amide NH of residue Asp168. These Hbond interactions are in fully agreement with previous X-ray crystal results reported before, allowing us to state that docking protocol used was effective for reproducing all key features already seen by other authors in the p38 kinase system.[33]

Please insert Figure 1 around here

On the other hand, all the inhibitors for the Aurora A data set were successfully docked within protein binding site, contrary to the previous docking results obtained by Lyne *et al*.[40] They reported that not all compounds were docked satisfactorily (2i – m) by the Glide module. The

weakest inhibitor did not yield a docking solution, and four of the inhibitors were docked with poor amide conformations in the DFG-out region of the binding site. Our docking results showed that all compounds adopted an extended conformation within Aurora A binding site, demonstrating the extent of the available binding pocket. Most of them established a hydrogen bond between the quinazoline N3 atom and backbone amide NH of residue Ala212. In some of the compounds, the amide carboxyl (located between the pyrimidine and benzoyl rings) plus one of the pyrimidine nitrogen atoms showed a bifurcated hydrogen bond to the conserved residue Lys161, while there were water-mediated contacts between other of the pyrimidine nitrogen atoms and the backbone amide NH of residue Asp273. The benzoyl moieties fitted into a hydrophobic pocket, which is occupied by Phe274 in the conserved DFG motif. Other Hbond interactions were observed at the entrance of binding pocket between Pr(morpholine) substituent and residues Arg136 and Arg219. All these protein-ligand interactions are in agreement with previous X-ray crystal studies on Aurora A kinase system reported by Heron *et al*.[34] The weakest inhibitor (2m) adopted a different conformation within Aurora A binding site due to its long structure that occupies all pocked and extends beyond to the solvent pocket.

It is worth to mention that CDK2 inhibitor series were obtained already in complex with the protein target from X-ray crystallographic data; and all inhibitors established the well-known Hbond interactions with the hinge region, and with other binding site conserved residues (Asp86, Lys33, Lys89, Gln131, Asp145), in a major or minor extent. All these observed Hbond interactions are in good agreement with previous structural studies based on huge X-ray crystallographic information available for CDK2 systems.[36–38]

Molecular Dynamic Simulations and Binding Site Hydrogen Bond Network Analysis.

All protein kinase – ligand complexes were submitted to equilibration and production MD simulations to relax the structural models as well as to include target flexibility and induce fit effects, respectively. This last aspect is extremely relevant in protein kinases which are particularly flexible proteins.[32] In Figure 2, the RMSD of the heavy atoms is plotted against the equilibration MD simulation time (2 ns) for the different protein kinase-ligand complexes (only the three most potent compounds in each series are presented), which reached a fairly good stability for CDK2 (top) after 0.5 ns at 1–1.2 Å, Aurora A (middle) after 0.5 ns at 1–1.4 Å and p38 (bottom) after 0.6 ns at 1–1.1 Å. Moreover, the evolution over time of the geometry of the main Hbond network formed by each inhibitor with key residues within the protein kinase binding site was also monitored (see Table 1SI in Supplementary Information section). In general it was observed that the H-bond network distances were maintained for all inhibitors during the MD simulations with an average value around of 2.8 Å.

Please insert Figure 2 around here

The abovementioned Hbond network was first carefully characterized through scripting tools developed at our laboratory. These programming tools allowed us to obtain the stability (this means a percentage of occurrence in which the geometry of the Hbond interactions was optimal) of every Hbond interaction established between the inhibitors and the binding site residues; as can be seen in Figure 3, where CDK2 inhibitor NU6102 is shown as a representative example. The relevance of these tools in the MD trajectory analysis relies in the straightforward

identification, processing and classification of key non-covalent interactions established between protein and ligand (for instance the Hbonds analysis performed in this work, o even other interactions like cation- π), which come from huge structural data derived from long MD simulations. All data concerning the percentage of stability between inhibitors and key residues at protein kinases binding site are reported in Table 2SI in Supplementary Information section.

Please insert Figure 3 around here

Once the stability of every Hbond was determined, the next 10 ns long MD simulations were performed in order to obtain the conformational structures needed for MM-GBSA calculations. These production MD simulations were strictly a continuation of the equilibration ones (same simulation water box, restraints, T, V, etc.), and it is expected that all key molecular interaction features observed in the short 2ns MD simulations remain stable. According with results from interaction network analysis (see Table 2SI), the HBond interactions between all inhibitors and residues at hinge region (Glu81 and Leu83 in CDK2, Ala212 in AuroraA and Met109 in p38) are the most stable and lasting ones along the production MD simulations (with occurrence percentages that range between 62 – 100%), therefore they were selected in order to apply the interaction based selection method. Other interactions were established and characterized, but they were ignored in this case due to their lower stability or occurrence in all ligands. For instance, there exist several HBonds mediated by water molecules in most of the protein-ligand systems, but these water molecules are located in the solvent channel and not within inner binding pocket. Therefore, there is a continuous flow and dynamic change of water molecules in several sites of the molecular complexes, which in principle render them problematic to be

included in the selection analysis. Our group has already characterized this complex behavior of water molecules in other protein kinase ligand systems.[61]

Conformation Selection Analysis and Prime-MM-GBSA Calculations.

In Table 2 are shown the correlation coefficients (R^2) obtained for several subsets of frames taken from production MD simulations of protein – ligand complexes. Two structure selection methods were used to search for statistical correlations between computational and experimental binding free energies, namely random selection and H-bond network selection. As can be observed in all protein kinase – ligand complexes, the random selection method showed correlation coefficients varying from medium to very low quality. For instance, for p38 – ligand and Aurora A – ligand complexes, the R^2 values were around 0.26 and 0.46 for all the studied subsets of frames, which roughly means that only 26% and 46% of the experimental affinity can be explained with the computed ΔG values, respectively. For CDK2 – ligand complexes the R^2 values showed a mean of 0.74, which suggest a better estimation of the computed ΔG values and therefore their comparison with experimental ones was improved, when compared with the previous two cases. That data may suggest two important observations: first, through the random selection method could not be possible to get meaningful protein – ligand structures that contribute to a good estimation of computed binding free energy. This issue is clearly demonstrated by the fact that the inclusion of tenths or even hundreds of MD protein – ligand structures into the subset used for computing ΔG values does not have any significant impact in the statistical correlation obtained. And second, the starting protein – ligand structure to be used in the MD simulations and subsequent estimation of ΔG values, seems to play an important role

in the good estimation of ΔG values. It is worth to point out that CDK2 complexes came from X-ray data meanwhile the p38 and Aurora A complexes were obtained through molecular docking experiments.

Regarding the statistical correlations obtained from H-bond network clustering selection, the results are encouraging due to R^2, a direct comparison between experimental and computed ΔG values, showed values varying from medium to good quality. The p38 and Aurora A complexes showed an improvement in their statistical R^2 to values around 0.82 and 0.73 for the subset of 10 frames, respectively. On the other hand, the improvement in statistical R^2 for CDK2 complexes was less pronounced taking a maximum value of 0.81 for subset of 10 frames. The addition of further structures into the computation of the averaged ΔG does not improve significantly the statistical R^2 values except for the Aurora A complexes, which presented a R^2 value of 0.83 for 20 frames. The subsequent inclusion of more frames (subsets of 50 or more frames) for getting the computational averaged ΔG values was in detriment of obtaining better statistical correlations against experimental data in all protein kinase – ligand complexes. All these data may suggest that the H-bond-based selection method seems to be more useful in the collection and ordering of MD structures for calculation of ΔG values using the MM/GBSA method, as could be seen for the three protein kinase – ligand systems reported in this study.

In Figure 4 are shown the best statistical correlation plots obtained from the H-bond network selection method. In all the graphs, the computed binding affinity values for all compounds are presented as $\Delta\Delta G_{bind}$ values, which means that they are relative with respect to the more favorable ligand ΔG value. For CDK2 complexes, which ligands are chemically diverse, it can

be seen that the more and less active compounds were correctly ranked by MM/GBSA method. The difference in energy between them was about 32.5 kcal/mol. The rest of compounds could fit pretty well the correlation trend line except for 2FVD ligand, which if it is removed from the dataset the correlation is slightly improved to a R^2 value of 0.86. The present results are in decent agreement with those presented by Hobza *et al*.[39] that studied the same compound data set using quantum semi empirical methods. Those authors obtained a correlation coefficient of 0.87, but they used a PM6 - DH2 method that accurately covered the dispersion interaction and H-bonding in the abovementioned system. In that regard, our results are encouraging because they only rely upon a scoring function based on molecular mechanics, the solvation terms calculated with a continuum approximation, as they have been implemented in MM/GBSA method, and the H-bond network selection method proposed in this work. It is expected that inclusion of such energy terms and an enhanced representation of H-bonding network pattern in protein kinase – ligand systems, allowed us to get better correlations. In order to evaluate the impact of including the target flexibility through the use of MD simulations on protein kinase – ligand complexes, the correlation coefficient was also calculated upon the X-ray crystallographic structures of CDK2 complexes. As can be seen in Figure 4 (top graph, closed red circles and red trend line), the R^2 value obtained was about 0.64, which roughly means that correlation coefficient for this comparison could be actually improved by including the flexibility of the target and rationalizing the selection of frames for ΔG estimation.

According to previous results, the p38 data set[33] studied here provides a number of challenges. The inhibitors bind to a p38 protein in a DFG-out conformation that makes possible that the ligands could bind to an additional hydrophobic pocket that is not present in the DFG-in protein

configuration. The substitution pattern around the middle phenyl ring of the compounds corresponds to subtle changes in structure that have a dramatic effect on the measured biological activities and the range of IC_{50} is quite small (10 nM to 2.1 μM), which is typical of the range being considered during lead optimization stages of a project.[40] Our computational protocol could rank correctly the more and less active compounds in this congeneric series; and moreover, the obtained statistical correlation (R^2 = 0.82) was better than the reported ones in similar previous studies (See Figure 4, middle). For instance, Lyne *et al*.[40] and Rapp *et al*.[60] found that statistical correlations between computed and experimental binding free energies, using their own developed computational protocols, were 0.71 and 0.70, respectively. Interestingly, the latter authors stated that allowing receptor flexibility in the test cases reported reduced the correlations between computed and measured binding affinities, in some cases quite dramatically. This is opposing to our findings that could suggest that including receptor flexibility is important in the correct estimation of relative ΔG values. The last tested case was the Aurora A complexes (Figure 4, bottom), which was also studied previously by same abovementioned authors. In previous reports, the statistical correlations leaved some compounds out due to poor docked conformations (in Lyne's work they were able to dock only 8 out of 13) or skipped the less active compound (Rapp's work). For Aurora A kinase, our result of R^2 equals to 0.83 is better than those reported before by Lyne and Rapp (R^2 = 0.56 and R^2 = 0.63, respectively).[40, 60] Despite our computational protocol could not rank the more active compound accurately in this congeneric series, it could do it for the less active one; moreover, the difference in binding energy between the more active compound and the best one according to MM/GBSA scoring was only about 1.5 kcal/mol.

All abovementioned results may suggest that our computational protocol, which included a novel H-bond network selection method applied to the estimation of binding free energies through the MM/GBSA method, seems to be able to improve the results from the currently used scoring functions and moreover, it was able to explain about 82% of the data variability in the binding affinities for a series of congeneric as well as non-congeneric compounds in the protein kinases systems selected as test cases. Ongoing research work is being performed in our group in order to test this protocol in other protein – ligand systems and to extend the H-bond network selection in the prediction of binding affinities using hybrid calculation methods, namely QM/MM or ONIOM approaches.

CONCLUSIONS

In the present work a selection method, namely H-bond network-based selection, was presented. The method was used in the recruitment and ordering of protein kinase – ligand structures derived from long molecular dynamic trajectories that were further used to estimate binding free energies by means of the MM/GBSA approach. The overall protocol was composed by two scripting subroutines that performed separate tasks: first, these accomplished a systematic evaluation of the H-bond network between inhibitor and residues surrounding it, obtaining as a result the occurrence (as a percentage) and stability of H-bond network. Second, and taking only into account the most stable H-bonds, the routine evaluated the geometrical deviation of selected contacts (distance, angles or dihedrals) from ideal values (calculated by statistical mode along MD or given as user input) in all MD trajectory structures and further ranked them by means of a proposed scoring function. Finally, the computed ΔG average values were obtained from several subsets of ranked frames. ΔG average values were also obtained for subsets of same size, but selected by random selection.

The abovementioned computational selection protocol, in conjunction with molecular docking, MD simulations and the MM/GBSA method, was used in the ranking of several inhibitors with measured biological activity (K_i and IC_{50} values) against CDK2, p38 and Aurora A kinases, that were used as test cases. The correlation coefficient values (R^2) obtained for comparison between experimental and computed binding free energies, and their evaluation against R^2 values obtained from random selection method, suggested that inclusion of H-bonding geometrical parameters in the selection of frames for MM/GBSA calculations seem to be useful in obtaining better

statistical correlations than those previously reported. The method was applied to congeneric (p38 and Aurora A kinases) as well as to non-congeneric series (CDK2) of compounds, with starting protein – ligand structures that came from both X-ray experiment and molecular docking, for CDK2 and p38 and Aurora A kinases, respectively. The computational protocol successfully ranked both kind of compounds and moreover, it was evidenced the importance of including the target flexibility by means of MD simulation on protein – ligand complexes. The starting protein – ligand structure seemed to play a minimal role in the H-bond selection method, but it acquired some importance when the selection of frames was made randomly, mostly in structures derived from molecular docking experiments.

The proposed computational protocol is under testing in other protein – ligand systems in order to check its robustness and to introduce some improvements in the H-bond network analysis and selection scripts with the aim to get a straightforward and more accurate evaluation of H-bonds. Some other non-covalent protein – ligand interactions (i.e. cation – π) would be also included to improve the selection of MD frames for MM/GBSA binding free energy calculations.

FIGURES

Figure 1. Conformational overlay of compounds within binding site of p38 (top) and Aurora A (bottom) kinases. Protein-ligand complexes were obtained from molecular docking experiments using as starting structures the p38 (2bak) and AuroraA (2c6e) crystallographic data deposited in PDB.

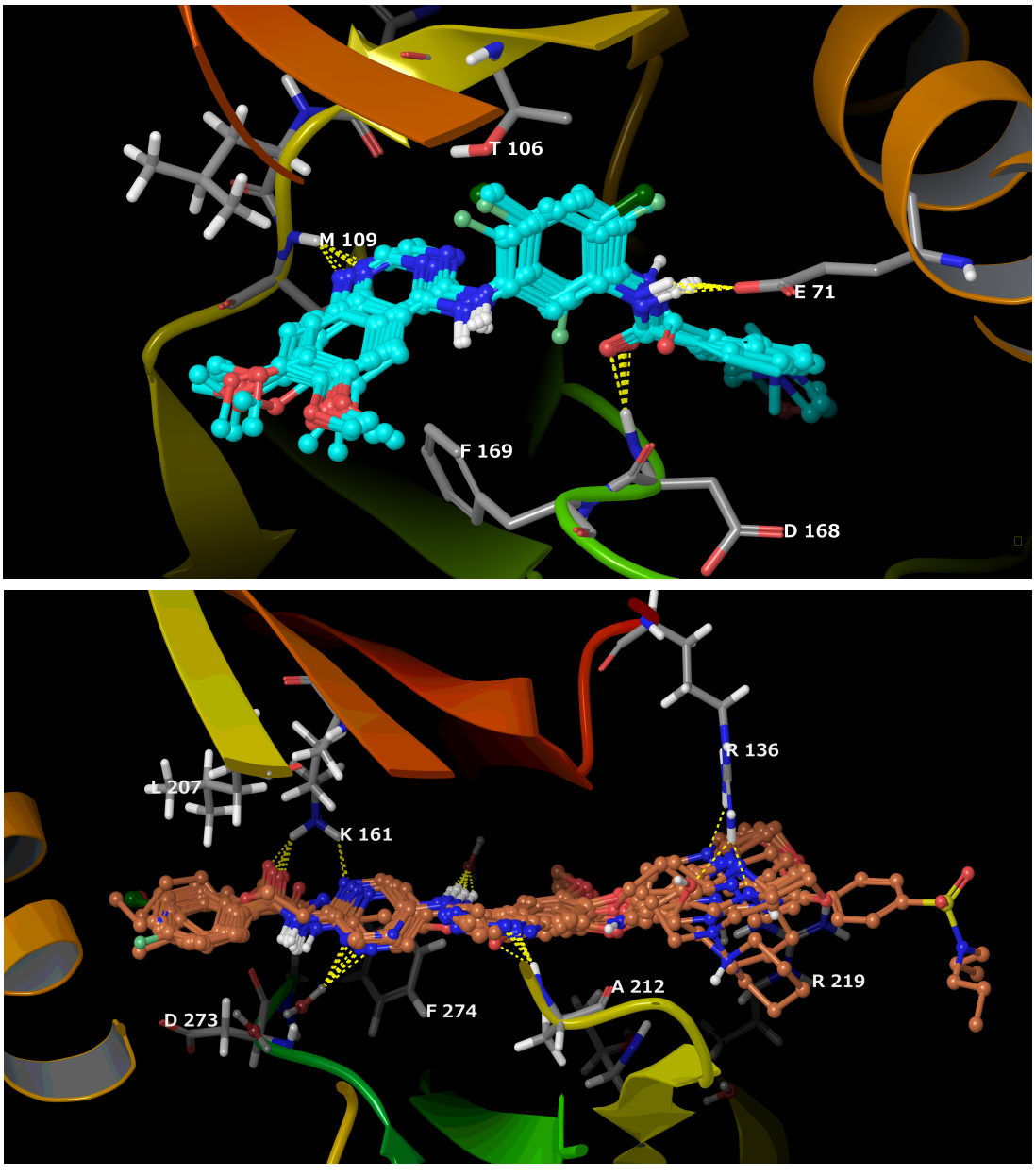


Figure 2. Plots of RMSD (Angstroms) values against simulation time (picoseconds). Data correspond to equilibration molecular dynamics of most potent compounds on the series studied. CDK2, Aurora A and p38 kinases are shown in graphs A, B and C, respectively.

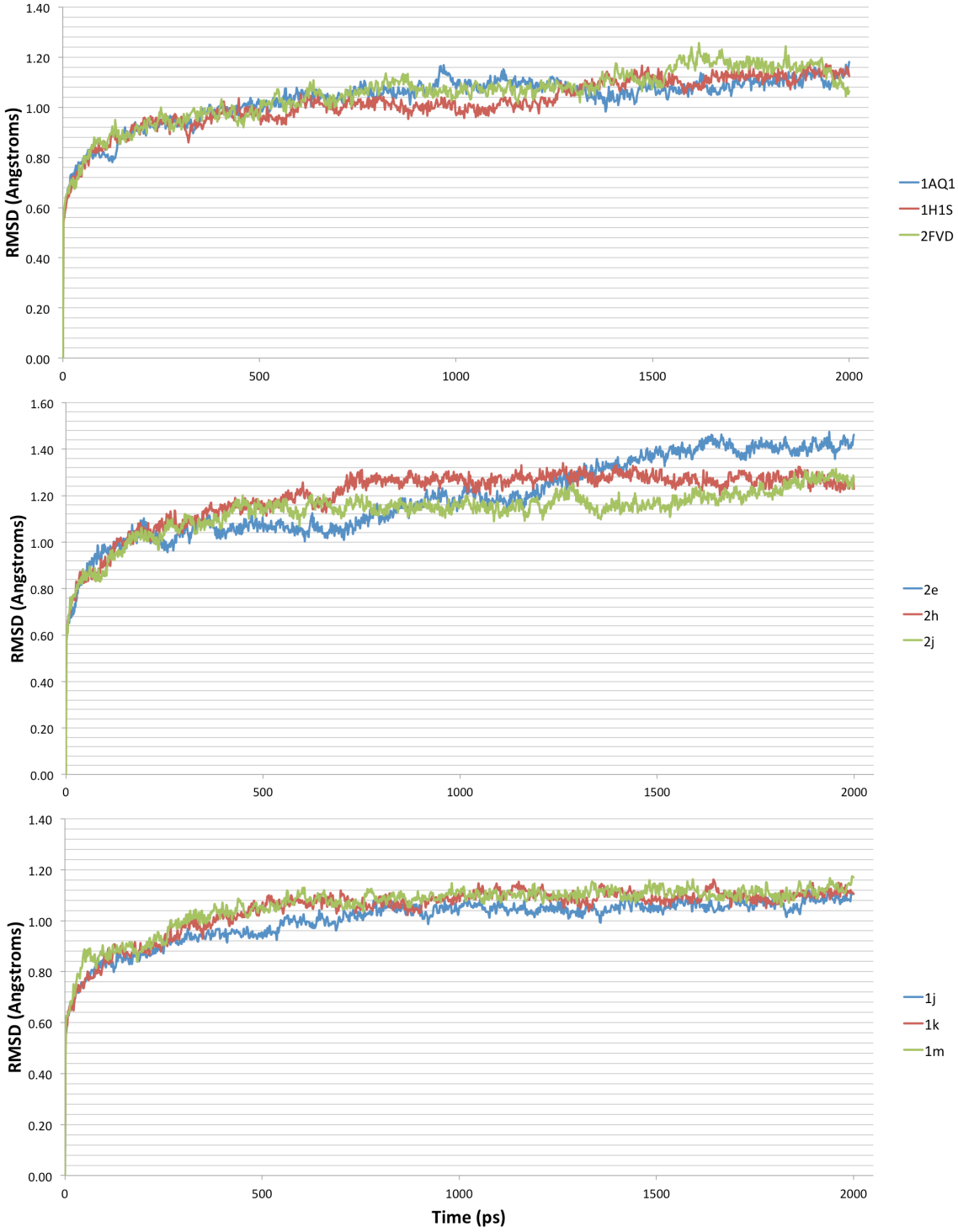


Figure 3. On the left, an atomic representation of all Hbond interactions (green arrows) established between CDK2 inhibitor NU6102 and key residues (and water) within protein binding site. On the right, a 2D schematic representation of the most stable (in percentage) Hbond interactions, between NU6102 and CDK2, identified along MD simulation time with the aid of developed scripting tools.

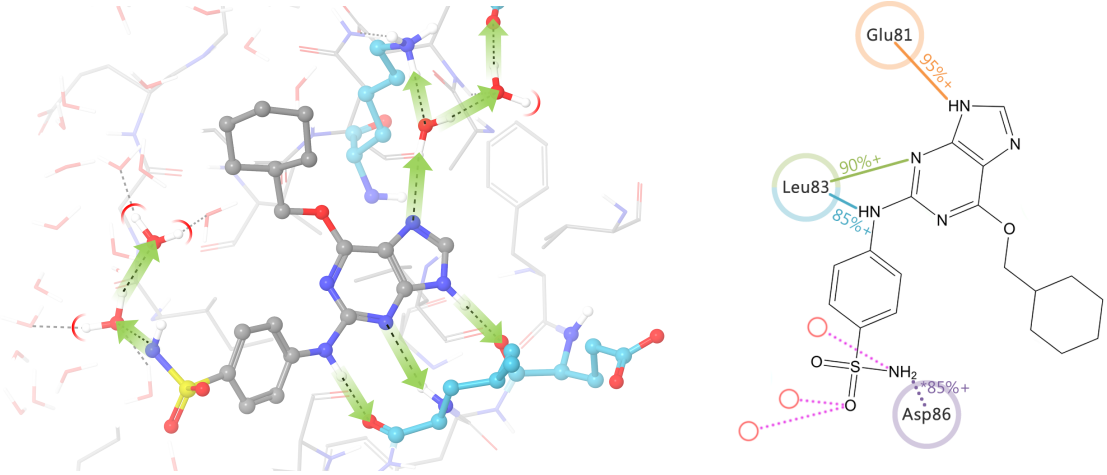
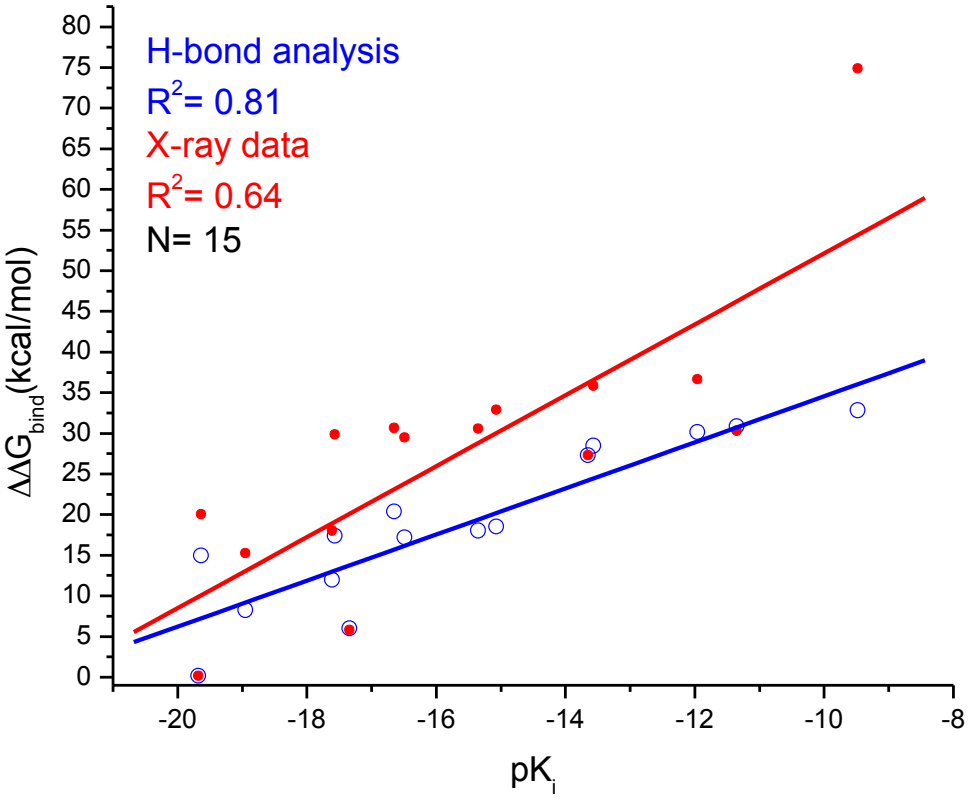
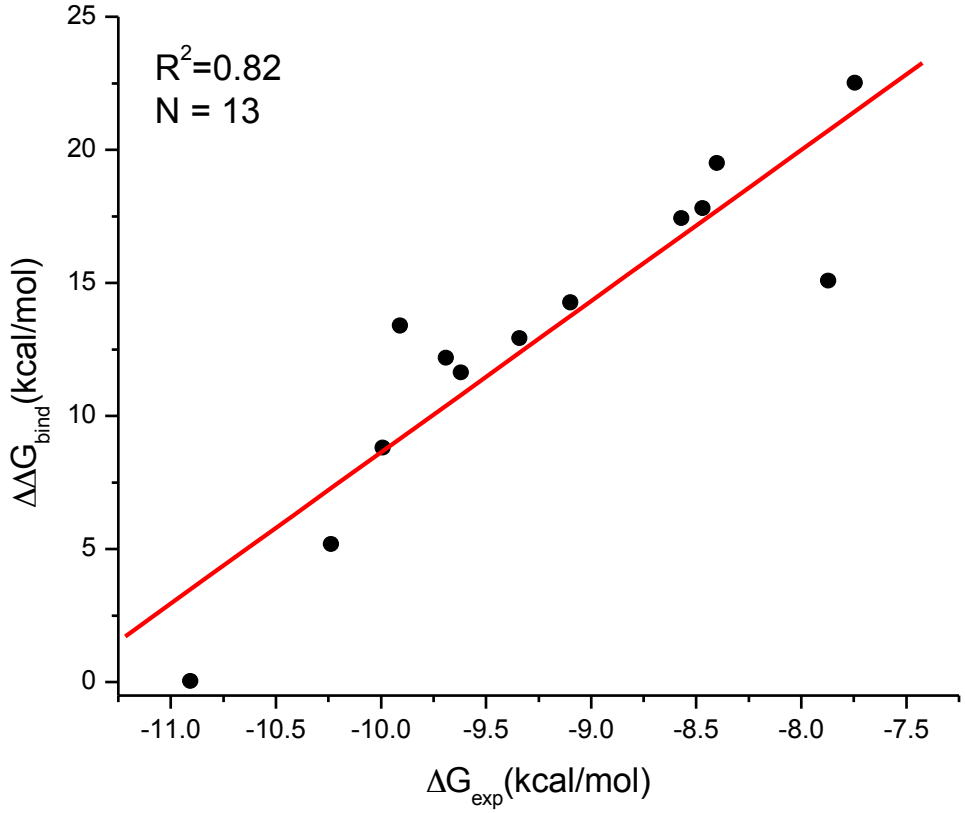
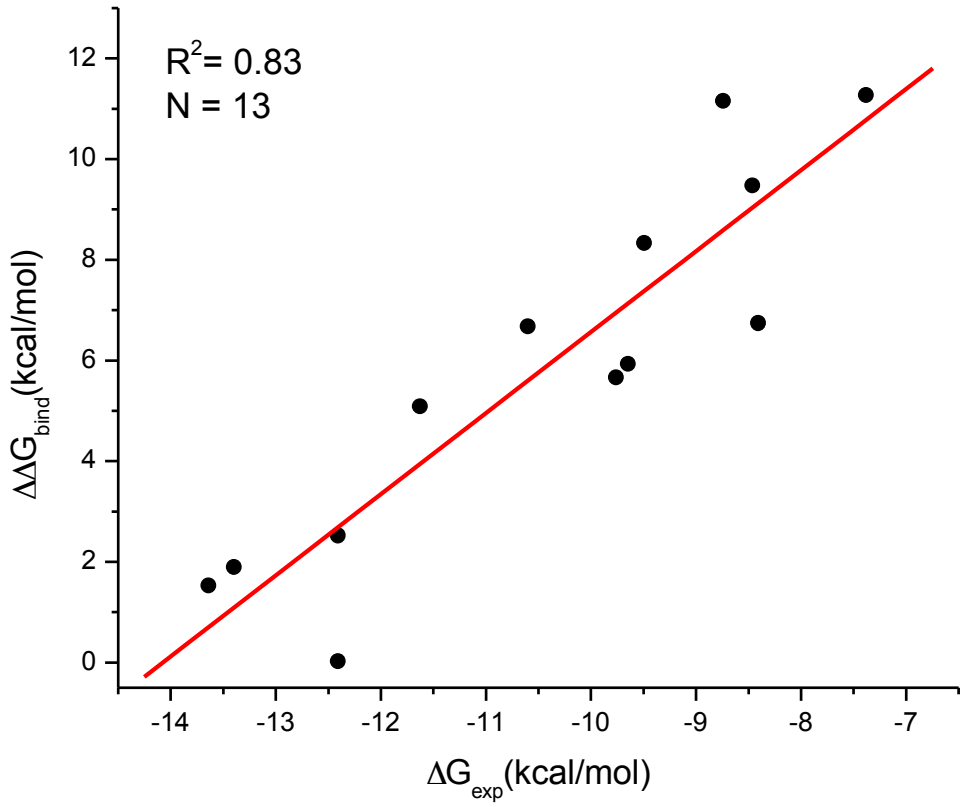


Figure 4. Best correlation plots obtained for comparison between calculated relative binding free energy and experimental biological activity expressed as LnK_i, in case of CDK2 inhibitors (top), or ΔG_{exp} for p38 and Aurora A inhibitors (middle and bottom, respectively), when structural-based clustering analysis was applied to all systems. For CDK2 plot is also included the correlation obtained when MM/GBSA was applied to protein ligand X-ray structures (red circles).







Scheme 1. Typical H-bond geometrical parameters included for defining the interactions between inhibitors and the studied protein kinases. The interactions between heterocyclic ring in a modeled inhibitor against residue Leu83 are taken as example.

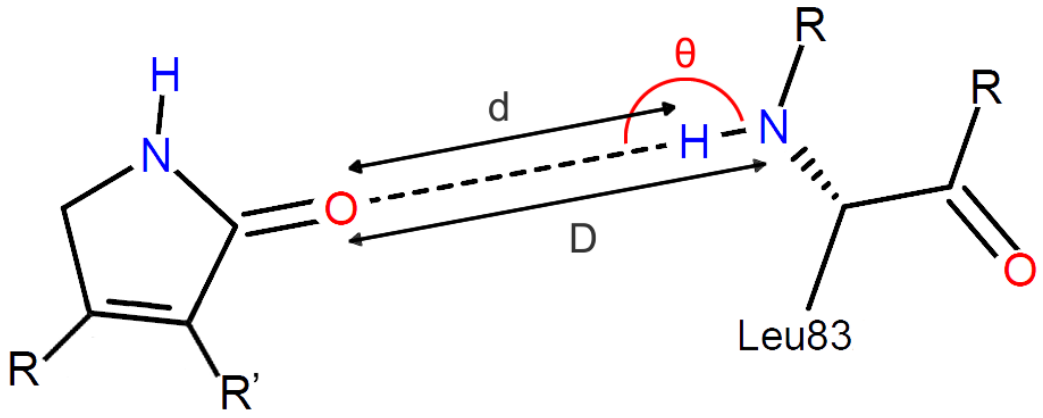


Table 1. Test set information

Targeted proteins			
Name	PDB id	Res.[a]	No. Ligands
p38 kinase	2bak	2.20	13
Aurora A kinase	2c6e	2.10	13
CDK2	1aq1, 1e1x, 1pkd*, 1pxj, 1pxl, 1pxm, 1pxn, 1pxp, 2fvd, 1h1p*, 1h1s*, 1ogu*, 2a4l, 2exm, 2x1n	2.00, 1.85, 2.30, 2.30, 2.50, 2.53, 2.50, 2.30, 1.85, 2.10, 2.00, 2.60, 2.40, 1.80, 2.75	15

[a] Res. (in Angstroms) is the experimental mean resolution of the respective X-ray structure.

* These structures contain the fully active form of CDK2. Only CDK2 with inhibitor was considered for calculations.

Table 2. Statistical correlation coefficients (R^2) obtained for comparison between experimental and computational binding free energies in protein kinase – ligand complexes. Two frame selection methods, and several subset frames, were used in the statistical comparison.

	Random selection						H-bond clustering selection					
# of frames	10	20	50	100	150	200	10	20	50	100	150	200
	Correlation coefficients (R^2)											
Aurora A	0.521	0.456	0.440	0.444	0.434	0.466	0.725	0.832	0.693	0.628	0.614	0.588
CDK2	0.741	0.758	0.742	0.743	0.726	0.738	0.810	0.807	0.796	0.788	0.784	0.782
p38	0.285	0.210	0.271	0.267	0.263	0.270	0.825	0.800	0.703	0.729	0.720	0.705

ASSOCIATED CONTENT

Electronic Supplementary Information (ESI) available: RMSD plots, as well as the averaged length of main H-bond interactions between ligands and test case protein kinases are reported along the equilibration molecular dynamics simulation (2ns). See DOI: 10.1039/b000000x/

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The manuscript was written through contributions from all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally in performing all computational calculations, programming tasks and revision of final manuscript. †Those

authors contributed equally in designing computational experiments and preparing and discussing the material to be included in final version of the manuscript.

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ABBREVIATIONS

CDK2, Cyclin Dependent Kinase 2; MD, Molecular Dynamics; Hbond, Hydrogen Bond; MM/GBSA, Molecular Mechanics/Generalize Born Surface Area.

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