Improving atom type diversity and sampling in co-solvent simulations using $\lambda$-dynamics

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Abstract

Co-solvent molecular dynamics (MD) simulations perform MD simulations of the protein in explicit water mixed with co-solvent molecules that represent functional groups of ligands potentially binding to the protein. The competition between different probes and water molecules allows the identification of the energetic preference of functional groups in different binding site moieties including enthalpic and entropic contributions. Co-solvent MD simulations have recently been applied to a variety of different questions in structure-based drug design but still have significant shortcomings. Among those issues is the limited chemical diversity of probe molecules ignoring the chemical context of the pharmacophoric feature represented by a probe. Here we present a novel co-solvent MD simulation method based on the $\lambda$-dynamics simulation concept that significantly increases the chemical diversity of functional groups investigated during co-solvent simulations. Application to four different test cases highlights the utility of the new approach to identify binding preferences of different functional groups and to correctly rank ligand series that differ by their substitution patterns.
1 Introduction

In recent years, co-solvent molecular dynamics (MD) simulations have become an important element in the toolbox of structure-based drug design methods. In co-solvent simulations the protein of interest (Fig. 1, orange) is simulated in a box of explicit water molecules (blue) mixed with other small organic molecules (red) as co-solvent. During the simulation, water and co-solvent molecules compete for pockets on the protein surface. Co-solvent simulations can be utilized for identifying allosteric or cryptic sites, assessing druggability of binding sites, identifying binding hotspots, and scoring and ranking ligands.

Figure 1: Scheme of co-solvent MD simulations. Left: MD simulations are performed for the protein of interest (orange) in a box of explicit water molecules (blue) mixed with other small organic molecules (red) as co-solvent. During the simulation, water and co-solvent molecules compete for pockets on the protein surface. (Right) A 3D grid is used to measure the frequency a co-solvent molecule occupying each position in the simulation box during the MD simulation (green grid boxes = high occupancy of a selected co-solvent molecule type). High frequency occupancy is directly related to more favorable interactions. Those occupancy grids can be used to identify hot spots of binding for each functional group represented by the co-solvent molecules and for estimating the free energy of binding.

One critical issue is that a balance between molarity of the co-solvent molecules and water needs to be obtained. A too high molarity of hydrophobic co-solvent molecules can result in denaturation of the protein and furthermore requires the use of artificial repulsion forces between co-solvent molecules to reduce aggregation of those co-solvent molecules. Carlson and co-workers overcame this issues by reducing the molarity of these co-solvent molecules. This reduction in molarity can be achieved by reducing the number of molecules per co-solvent type, but results in very slow convergence rates for sampling the preference of each probe to interact with the protein in different spatial locations. In contrast, in all
previous co-solvent approaches, only a relatively small set of different types of co-solvent probes is used per simulation to allow for sufficient convergence throughout the sampling process.\textsuperscript{2,9,15,16} This small set of probes is used to represent general pharmacophoric features of potentially interacting ligands, such as aromatic, aliphatic, hydrogen-bond donor, hydrogen-bond acceptor, and charged groups. The underlying hypothesis is that those pharmacophore features are able to represent all important types of interactions between protein and ligand atoms.

Figure 2: Comparison of density grids of physicochemically related co-solvents simulated at human cathepsin L (hCatL) (PDB-code: 2yj8). Differently colored grids represent different co-solvent molecules used: methanol (yellow), ethanol (blue), isopropanol (red). Simulations were performed with 0.25 M for each co-solvent molecule for 100 ns using OpenMM. Densities of hydroxyl oxygen atom are shown at an isolevel corresponding to 100-fold increase over average density in bulk solvent.

This assumption, however, neglects the chemical context of the functional group that can have significant influence on its interaction preference. Thus, different co-solvent molecules representing the same general interaction type display different occupancy distributions due to the detailed interaction profile with the protein. Analyzing the distribution of the same atom type in different molecular probes, e.g. hydrogen-bond donor/acceptor of the hy-
droxyl group in methanol, ethanol and isopropanol (Fig. 2) shows that in some moieties the hydroxyl-group densities of the different probes overlap (blue arrow) but that in other regions significant variability among the different probes exists (red arrow). This demonstrates that the occupancy of the same atom type differs dependent on the chemical context of the atom type.

Therefore, the limited variety of types of probes in standard co-solvent simulations is unable to represent the large spectrum of atom types in ligands potentially binding to a target protein. This led to the recognition that the proper selection of co-solvent molecules for each drug design project is a critical challenge for applications of co-solvent simulations.\textsuperscript{1}

Here, we present a novel co-solvent method to largely overcome this issue (Fig. 3). To allow for diverse representation of potential ligands functional groups without denaturation of the protein we have developed a new form of co-solvent simulations based on $\lambda$-dynamics.\textsuperscript{17–20} Similar types of co-solvent probes are combined into a single probe group (Fig. 4). Each probe group represents a set of similar but distinct co-solvent molecules. The contribution of each probe $i$ from probe group $p$ to the overall energy of the simulation system is determined by a coefficient $\lambda_{p,i}$. $\lambda_{p,i}$ can also be viewed as the probability of finding a specific molecule $i$ among the co-solvent group $p$ at the current simulation time point at the current location. At the beginning of a simulation, all members of a probe group have equal probability. Those probabilities change throughout the simulation largely based on their interaction strength with the environment, i.e. probe $i$ with better interaction energy with its surrounding compared to probe $j$ from the same probe group $p$ will observe an increase in its $\lambda_{p,i}$-value compared to $\lambda_{p,j}$. This process optimizes the interactions of the probes with the protein on-the-fly, consequently sampling a large multitude of different co-solvent molecules at low molarity.
Figure 3: Scheme of proposed λ-dynamics co-solvent simulation technique to model larger spectrum of diverse probes compared to standard co-solvent simulations. The figure displays the example of modeling different substituted benzene probes. In λ-dynamics simulations each probe represents all six different substitutions (right) where the contribution of each substitution to the overall potential and kinetic energy of the system is determined by a dynamically changing coefficient λ that varies dependent on the probes interaction strength with its surrounding. Here, darker gray colors and thicker lines represent larger λ value. Simulating all six probes separately in standard co-solvent simulations (left) would require a six-fold reduction in number of entities per probe, thus reducing sampling convergence. Alternatively a six-fold increase in total number of probes would significantly increase co-solvent density (inducing problems such as aggregation and denaturation) or increasing the number of water molecules and thus the size of simulation system by a factor of approximately six.
2 Material and Methods

2.1 λ-Dynamics Co-Solvent Simulations

A novel concept for co-solvent simulations is devised to overcome the previously discussed issue of the lack of variety in co-solvent molecules. As discussed in the Introduction, a balance between molarity of the co-solvent molecules and water need to be achieved. To prevent protein denaturation, aggregation of hydrophobic co-solvent molecules and to increase the signal-to-noise ratio, we use a relatively low co-solvent fraction of 0.25 M per probe type similar to the studies of Carlson, MacKerrel and co-workers.1,9,12,14,15 At the same time, we aim to sample a diverse set of functional groups found in drug-like molecules. To allow for sufficient sampling of a large spectrum of functional groups at relatively low co-solvent concentrations in simulation times comparable to standard co-solvent simulations (typically few hundreds ns), the advanced sampling techniques of λ-dynamics is incorporated.17–20

In λ-dynamics, each co-solvent molecule \( p \) is a linear combination of all co-solvent molecules (=copies) \( i \) within its own group (Fig. 4, each row represents one group). All copies \( i \) of the same molecule \( p \) do not interact with each other and contribute with a factor of \( \lambda_{p,i} \in [0; 1] \) to the overall interaction with other co-solvent molecules, water and protein residues. The
sum of $\lambda_{p,i}$ values is one. The system is propagated using MD with the extended potential energy

$$V(X, \{x\}, \{\lambda\}) = V_{\text{env}}(X) +$$

$$\sum_{p=1}^{N_{cs}} \sum_{i=1}^{n_p} \lambda_{p,i} (V(X, x_{p,i}) - F_{p,i}) +$$

$$\sum_{p=1}^{N_{cs}-1} \sum_{i=1}^{n_p} \sum_{q=1}^{N_{cs}} \sum_{j=1}^{n_q} \lambda_{p,i} \lambda_{q,j} V(x_{p,i}, x_{q,j})$$

with $F_{p,i}$ being the bias, representing the desolvation free energy of a copy $i$ of co-solvent $p$, $x_{p,i}$ the coordinates of the individual co-solvent, $X$ the coordinates of the remainder of the system including its potential energy function $V_{\text{env}}$, $N_{cs}$ the total number of co-solvent groups, and $n_p$ the number of probes within each co-solvent group $p$. $\lambda_{p,i}$ are additional degrees of freedom that are propagated every time step by solving Lagrange’s equation. Whereas the first term on the right-hand side describes the interaction between all atoms except the copied probes, the second term describes the interactions between copied probes and environment, and the third term the interactions among the copied probes.

Figure 5 shows the propagation for one exemplary co-solvent probe group $p$ consisting of ethanol ($i = 0$) and aminomethanol ($i = 1$) that is entering a small protein pocket. Initially being a full ethanol molecule, the $\lambda_{p,0}$ being one, the interaction between probe group and protein prefers the aminomethanol copy of the same group, represented by $\lambda_{p,1}$. During the $\lambda$-dynamics simulation $\lambda_{p,0}$ therefore shifts towards a value of zero and $\lambda_{p,1}$ towards one.

Instead of propagating $\lambda$ variables directly, we use the transformation of $\lambda$ into $\theta^{20}$

$$\lambda_{p,i} = \frac{\exp(3.5 \sin(\theta_{p,i}))}{\sum_{i=1}^{n_p} \exp(3.5 \sin(\theta_{p,i}))}$$

which automatically fulfills the holonomic constraints that the sum of $\lambda_{p,i}$ within a probe group has to be one. The additional variable $\theta$ with fictitious mass $m_\theta = 5$ is propagated
Figure 5: Example for potential transition between two different copies (ethanol and aminomethanol) of a probe group in $\lambda$-dynamics simulation.
using the potential energy defined in equation 1 using Lagrange’s equation:

\[
\frac{d}{dt} \frac{\partial L}{\partial \theta_{p,i}} = \sum_{j=1}^{n_p} \frac{\partial L}{\partial \lambda_{p,j}} \frac{\partial \lambda_{p,j}}{\partial \theta_{p,i}}
\]

with \( L = T_X + T_{\{x\}} + T_{\{\theta\}} - V(X, \{x\}, \{\lambda(\theta)\}) \)

where \( T_X, T_{\{x\}}, T_{\{\theta\}} \) are the corresponding kinetic energies of the various degrees of freedom.

### 2.2 Calculation of Bias

1. Simulate individual probe in box of water using standard co-solvent simulations

2. Simulate all probes in box of water using \(\lambda\)-dynamics co-solvent simulations

\[ \rho_{p,0} \approx \rho_{p,1} \approx \rho_{p,2} \approx \ldots \approx \rho_{p,n} ? \]

The bias \( F_{p,i} \) in equation 1 represents the desolvation energy of a probe or more precisely
the energy of probe \( i \) in probe group \( p \) in a simulation system containing all probes used in the co-solvent simulation without the presence of a protein. To calculate the bias for each probe in a simulation, we developed a 2-step process (Fig. 6): First, each probe is simulated in isolation in a small box of explicit water molecules and its energy (internal energy plus interaction energy with surrounding water molecules) is averaged over a 1 ns simulation. This initial bias is used in a second, subsequent step, where the full set of co-solvent probes is simulated. In this step, each probe group with concentration of 0.25 M is added to a box of explicit water molecules. This set of probe groups in water is simulated using \( \lambda \)-dynamics co-solvent simulations for 2 ns. The population of each probe \( i \) within a probe group being the dominant copy (here: \( \lambda_{p,i} > 0.8 \)) is computed over the trajectory. The bias \( F_{p,i} \) for all probes is adjusted after the simulation to enforce equal populations among a probe group, i.e. \( F_{p,i} \) is reduced if the population of \( i \), \( \rho_{p,i} \), is too large, and \( F_{p,i} \) is increased if \( \rho_{p,i} \) is too small. This iterative process is repeated until near-equal occupancies \( \rho_{p,i} \) for each copy \( i \) among all copies of probe group \( p \) is obtained throughout a 2 ns co-solvent-water simulation. This set of biases is used in subsequent \( \lambda \)-dynamics co-solvent simulations with protein. This guarantees that differences in occupancies of probe copies in the presence of the protein reflect the preference of the probe type to interact with the moiety of the protein with respect to the unbound, solvated state.

2.3 Implementation Details

We implemented the \( \lambda \)-dynamics co-solvent methodology in OpenMM including a CUDA implementation for GPU processing.\(^\text{21}\) The preparation of \( \lambda \)-dynamics co-solvent simulations was automated in form of a PyMOL plugin and utilizing Amber and Gromacs preparation tools.\(^\text{22,23}\) First, probes are added around the protein using Gromacs’s insert-molecules utility and subsequently water is added to fill the simulation box using Amber’s tleap program. Co-solvent simulation-specific parameters such as probe types, probe density, and restraints can be specified within the PyMOL plugin.
New variables and parameters associated with \( \lambda \)-dynamics co-solvent simulations were defined and added to the OpenMM simulation using a modified Python API. \( \lambda \)-dynamics propagation and force calculation routines were added or modified directly on the C++ and CUDA level of OpenMM. Restraints between co-solvent molecules within the same probe group, repulsive forces between aliphatic probes to prevent aggregation and virtual sites for extra particles representing halogen atom’s sigma holes are added throughout the preparation process to the Amber topology files using parmed and to the OpenMM implementation using the Python API.

2.4 Test Systems and Probe sets

We applied \( \lambda \)-dynamics co-solvent simulations to four protein systems each starting from a selected holo protein structure: Human cathepsin L (hCatL; PDB-code: 2yj2), Y220C mutant of the p53 tumor suppressor (p53-Y220C; 4agl), X-linked inhibitor of apoptosis protein (XIAP; 5c7a), and HIV-1 protease (2i0d).

2.4.1 Human cathepsin L (hCatL)

The first test system contains a series of ligands binding to hCatL (Fig. 7).\textsuperscript{24,25} The compounds share a common scaffold and differ only in the substitution pattern (halogenated, methylated, and unsubstituted analogs) on the terminal benzene ring binding to the S3 subpockets. Significant differences in affinity are observed for substitutions on the para-aryl-X position which can interact with the backbone carbonyl oxygen of GLY-61. Affinity increases upon changing the \textit{para}-aryl-X substituent from F, over H, CH\(_3\), Cl, Br, to I, consistent with the potential formation of halogen bonds between Cl, Br, and I substituted compounds at position X with GLY-61.

To test if \( \lambda \)-dynamics co-solvent simulations are feasible to differentiate between the interaction strengths of different substitution patterns of the hCatL ligand series, we added the probe group containing different benzene-derived probes (benzene, fluoro-benzene, chloro-
Inhibitors of hCatL undergo reversible covalent binding to the thiol group of CYS 25 in the S1 pocket under formation of thioimidates. In addition, hydrogen bonds are also formed to the backbone NH group of GLY 68, and C=O group of ASP 162 (green dashed lines). The halogen-bonding interaction to GLY 61 is highlighted in red.

benzene, bromo-benzene, iodo-benzene, and methyl-benzene) to a standard set of co-solvent molecules containing acetaldehyde, formamide, and propane (Table 1).

Table 1: List of co-solvent probes used for test system hCatL and p53-Y220C.

<table>
<thead>
<tr>
<th>Probe group</th>
<th>Co-solvent molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Benzene</td>
</tr>
<tr>
<td></td>
<td>F-benzene</td>
</tr>
<tr>
<td></td>
<td>Cl-benzene</td>
</tr>
<tr>
<td></td>
<td>Br-benzene</td>
</tr>
<tr>
<td></td>
<td>I-benzene</td>
</tr>
<tr>
<td></td>
<td>CH₃-benzene</td>
</tr>
<tr>
<td>2</td>
<td>Propane</td>
</tr>
<tr>
<td>3</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>4</td>
<td>Formamide</td>
</tr>
</tbody>
</table>

For a more precise modeling of the electrostatic interactions of chloro-, bromo-, and iodo-aryl-containing molecules in context of a molecular mechanics representation, an extra-point (EP) of positive charge was added to mimic the electron-deficient outer lobe of the half-filled \( p_z \) orbital of the halogen atom, also known as \( \sigma \)-hole.\(^{26-29}\) Following a previous study of
Jorgensen and Schyman\textsuperscript{28} this massless dummy atom was placed along the C-Cl, C-Br and C-I bond at a 1.6 Å, 1.6 Å, 1.8 Å distance from the halogen atom, respectively (Figure 8 and Table 2). Restrained electrostatic potential (RESP) procedure with the ESPGEN program within the Amber suite\textsuperscript{23} was used to fit the partial charges to the electrostatic potential calculated using Gaussian16.\textsuperscript{30} The quantum-mechanical calculations were performed at the HF/6-31G* level with iodine atoms treated with the aug-cc-pVDZ-PP basis set. Additional force field parameters for the probes were derived using the general AMBER force field (GAFF).

![Figure 8: Extra-point (EP) in chloro-, bromo-, and iodo-benzene probes to model σ-hole.](image)

Table 2: RESP-fitted partial charges of different benzene probes used in the co-solvent simulation. X column for CH\textsubscript{3}-benzene specifies carbon and hydrogen partial charges of methyl group.

<table>
<thead>
<tr>
<th>Atom name</th>
<th>EP</th>
<th>X</th>
<th>C3</th>
<th>C2/C4</th>
<th>C1/C5</th>
<th>C6</th>
<th>H2/H3</th>
<th>H1/H4</th>
<th>H5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>N/A</td>
<td>-0.130</td>
<td>-0.130</td>
<td>-0.130</td>
<td>-0.130</td>
<td>-0.130</td>
<td>0.130</td>
<td>0.130</td>
<td>0.130</td>
</tr>
<tr>
<td>F-benzene</td>
<td>N/A</td>
<td>-0.257</td>
<td>0.475</td>
<td>-0.338</td>
<td>-0.070</td>
<td>-0.235</td>
<td>0.190</td>
<td>0.147</td>
<td>0.158</td>
</tr>
<tr>
<td>Cl-benzene EP</td>
<td>0.064</td>
<td>-0.313</td>
<td>0.324</td>
<td>-0.236</td>
<td>-0.126</td>
<td>-0.156</td>
<td>0.180</td>
<td>0.149</td>
<td>0.147</td>
</tr>
<tr>
<td>Br-benzene EP</td>
<td>0.103</td>
<td>-0.359</td>
<td>0.309</td>
<td>-0.218</td>
<td>-0.137</td>
<td>-0.145</td>
<td>0.178</td>
<td>0.150</td>
<td>0.146</td>
</tr>
<tr>
<td>I-benzene EP</td>
<td>0.113</td>
<td>-0.360</td>
<td>0.270</td>
<td>-0.197</td>
<td>-0.157</td>
<td>-0.118</td>
<td>0.177</td>
<td>0.153</td>
<td>0.142</td>
</tr>
<tr>
<td>CH\textsubscript{3}-benzene</td>
<td>N/A</td>
<td>C: -0.382</td>
<td>0.287</td>
<td>-0.279</td>
<td>-0.106</td>
<td>-0.179</td>
<td>0.157</td>
<td>0.136</td>
<td>0.140</td>
</tr>
</tbody>
</table>

\subsection*{2.4.2 Y220C mutant of the p53 tumor suppressor (p53-Y220C)}

The second test system contains a series of ligands binding to p53-Y220C (Fig. 9).\textsuperscript{31,32} The compounds share a common scaffold and differ in the substitution pattern on the terminal benzene ring. Similar to hCatL, significant differences in affinity are observed for halogen
substitutions, here on the meta-aryl positions. Affinity increases upon changing the meta-aryl-Y substituent from Cl, over Br, to I, consistent with the potential formation of halogen bonds between Cl, Br, and I substituted compounds at position Y with LEU 145. The same probe set (Table 1) as for hCatL was used for p53-Y220C.

Figure 9: Structures of p53-Y220C mutant stabilizers and their dissociation constants.\textsuperscript{31,32} Water-mediated hydrogen bonds are formed to the backbone groups of VAL 147 and ASP 288 (green dashed lines). The halogen-bonding interaction to LEU 145 is highlighted in red.

2.4.3 X-linked inhibitor of apoptosis protein (XIAP)

The third test system contains a series of ligands binding to the BIR3 domain of X-linked inhibitor of apoptosis protein (XIAP-BIR3) (Fig. 10).\textsuperscript{33} The compounds share a common scaffold and differ in the substitution pattern on the terminal indoline ring. The observed SAR depends strongly on the electronic properties of the indoline ring, with electron-poor rings being more potent than electron-rich rings. Binding affinities correlate well with the Hammett substituent constants.\textsuperscript{33} A different probe set (Table 3) compared to hCatL and p53-Y220C was used to demonstrate the generality of our \( \lambda \)-dynamics co-solvent approach.
Figure 10: Structures of inhibitors binding to the BIR3 domain of X-linked inhibitor of apoptosis protein (XIAP-BIR3) and their IC$_{50}$ values. Hydrogen bonds and water-mediated hydrogen bonds are formed to THR 308, GLU 314, GLN 319, and TRP 323 (green dashed lines).

Table 3: List of co-solvent probes used for test system XIAP.

<table>
<thead>
<tr>
<th>Probe group</th>
<th>Co-solvent molecules</th>
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<td>1</td>
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<td></td>
<td>Cl-benzene</td>
</tr>
<tr>
<td></td>
<td>Br-benzene</td>
</tr>
<tr>
<td></td>
<td>CH$_3$-benzene</td>
</tr>
<tr>
<td></td>
<td>CF$_3$-benzene</td>
</tr>
<tr>
<td></td>
<td>OCH$_3$-benzene</td>
</tr>
<tr>
<td></td>
<td>NH$_2$-benzene</td>
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<tr>
<td>2</td>
<td>Propane</td>
</tr>
<tr>
<td>3</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>4</td>
<td>Formamide</td>
</tr>
</tbody>
</table>

2.4.4 HIV-1 protease

The fourth test system contains a series of ligands binding to HIV-1 protease (Fig. 11). The compounds share a common scaffold and differ in the substitution pattern on the two terminal benzene rings. This example tests the feasibility of λ-dynamics co-solvent simulations to identify the likelihood of functional groups in distinct moieties of the binding site of a target.
protein. A third probe set (Table 4) was used to demonstrate the generality of our \(\lambda\)-dynamics co-solvent approach.

![Figure 11: Structures of HIV-1 protease inhibitors and their inhibition constants.](image)

Hydrogen bonds and water-mediated hydrogen bonds are formed to ASP 25A and B, ASP 29A, ASP 30B, ILE 50A and B (green dashed lines). Ac=acetate.

**Table 4: List of co-solvent probes used for test system HIV-1 protease.**

<table>
<thead>
<tr>
<th>Probe group</th>
<th>Co-solvent molecules</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td></td>
<td>F-benzene</td>
</tr>
<tr>
<td></td>
<td>CF(_3)-benzene</td>
</tr>
<tr>
<td></td>
<td>OCH(_3)-benzene</td>
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<td>OCF(_3)-benzene</td>
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<td>NH(_2)-benzene</td>
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<td>2</td>
<td>Propane</td>
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<tr>
<td>3</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>4</td>
<td>Formamide</td>
</tr>
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</table>

**2.5 Simulation Settings**

MD simulations were performed using our modified version of the GPU-accelerated OpenMM\(^{21}\) package with the AMBER14SB force field\(^ {35}\) and SPC/E water model.\(^ {36,37}\) Bonds containing hydrogen atoms were constrained to their equilibrium length using the SHAKE algorithm.\(^ {38}\)
Long-range electrostatic interactions were treated with the Particle Mesh Ewald method\textsuperscript{39} with a cutoff of 10 Å for direct interactions. The Lennard-Jones interactions were truncated at a distance of 10 Å, and a long-range isotropic correction was applied for Lennard-Jones interactions beyond the cutoff. Langevin integration with a time step of 2 fs was used for propagating the system. Temperature coupling was modeled with a stochastic thermostat with collision frequency of 1 ps\textsuperscript{-1}. Pressure control was guaranteed by adjusting the size of the periodic box using a Monte Carlo approach. The system was first energy minimized and then equilibrated at a temperature of 300 K for 200 ps MD simulation length at constant volume (NVT), followed by 400 ps of equilibration at a temperature of 300 K and pressure of 1 bar (NpT). Production runs were performed for 10 ns. All simulations are run with periodic boundary conditions in all three dimensions. A weak restraint on the backbone heavy atoms with a force constant of 50 kJ mol\textsuperscript{-1} nm\textsuperscript{-2} was applied in all simulations. In order to prevent relative diffusion of probe copies of the same probe group, harmonic distance restraints with a force constant of 50000 kJ mol\textsuperscript{-1} nm\textsuperscript{-2} were added between centroid atoms of the corresponding probe copies. For benzene derivatives a virtual site was added in the center of the aromatic ring. This virtual site was used for restraining copies of the same probe group. 50 different simulations with different initial co-solvent positions were performed.

Similar to Guvench and MacKerell,\textsuperscript{2} an artificial repulsive force defined by a Custom-NonbondedForce in OpenMM\textsuperscript{21} (Equation 4) was added between the center of an aromatic ring and/or the centroid of propane to prevent the aggregation between hydrophobic probes.

\begin{equation}
V(r) = \begin{cases} 
V_0(r - 7\text{Å})^2 & \text{if } r \leq 7 \text{ Å} \\
0 & \text{if } r > 7 \text{ Å}.
\end{cases}
\end{equation}

with $V_0 = 200$ kJ mol\textsuperscript{-1} nm\textsuperscript{-2}.
2.6 Analysis of $\lambda$-Dynamics Co-Solvent Simulations

A 3D grid with grid spacing of 0.5 Å was placed over the simulation box. The probe occupancy for each atom type B on the 3D grid was collected every 1 ps throughout the production simulation. Only probe copies with $\lambda > 0.8$ contribute to the occupancy at a given MD snapshot. Averaging the atom type occupancy over all 50 simulations, density maps were obtained for each atom type as specified in Table 5.

These atom type density maps could then be converted to free energy maps (FEMaps) using classical inverse Boltzmann equation with respect to the bulk reference state,

$$\Delta G_B(i) = -k_B T \ln \left( \frac{P_B(i)}{P_B(\text{bulk})} \right)$$

For each density map, the grid occupancies $P_B(i)$ obtained from co-solvent simulations were normalized by the bulk occupancy $P_B(\text{bulk})$. $P_B(\text{bulk})$ was determined from $\lambda$-dynamics co-solvent simulations using the same set of probe groups without the presence of the protein (see Section 2.2).

This approach assumes that the atom type densities are sampled separately from each other. This assumption, however, is incorrect as the densities of two atom types located on the same co-solvent molecule are highly correlated. For example, the fluorine density of fluorobenzene probe in a specific binding site location is strongly influenced by the interaction strengths of the benzene group of the same probe molecule with the protein. Therefore, a fluorine density near the backbone carbonyl oxygen of GLY 61 higher than bulk will be observed in hCatL (Figure 7) if the aromatic ring of fluorobenzene interacts favorably with the hydrophobic residues in the S3 subpocket.

Thus, equation 5 needs to be modified to acknowledge the correlation between different atom types on the same probe molecule. Let us assume that a probe consists of two different atom types A and B with energy values $\epsilon_i$ and $\mu_i$ on each grid point $i$, respectively. The probability of sampling atom type B at a given grid point $i$ is then determined by
\[ P_B(i) = \frac{\sum_{k \in S_i} e^{-\beta(\mu_i + \epsilon_k)}}{\sum_{j} \sum_{k \in S_j} e^{-\beta(\mu_j + \epsilon_k)}} = \frac{\sum_{k \in S_i} e^{-\beta(\mu_i + \epsilon_k)}}{Z} \]  

with \( S_i \) specifying all grid points in the surrounding of \( i \) that are accessible to atom type A in the probe molecule, if atom type B is occupying grid point \( i \).

Solving equation 6 for \( \mu_i \)

\[ \mu_i = -k_B T \ln P_B(i) + k_B T \ln \left( \sum_{k \in S_i} e^{-\beta \epsilon_k} \right) - k_B T \ln Z \]  

and normalizing by the bulk reference state gives

\[ \Delta G_B(i) = -k_B T \ln \left( \frac{P_B(i)}{P_B(\text{bulk})} \right) + k_B T \ln \left( \frac{\sum_{k \in S_i} P_A(k)}{\sum_{k \in S_{i,\text{bulk}}} P_A(\text{bulk})} \right) \]

The second term of the right side of equation 8 corrects the free energy computation for atom type B by the densities of atom type A as part of the same probe molecule. To compute this correction factor, one hundred points equally distributed on the surface of a sphere with center at grid point \( i \) and radius equal to the bond length between atoms A and B on the probe molecule are selected. The densities of atom type A on these hundred points are accumulated and normalized by the corresponding bulk densities.

### 2.7 Convergence of Free Energy Maps Computation

For convergence studies, occupancy maps were also computed at 1, 2, 3, 4, 5, 7 ns intervals of each of the 50 simulations. The 50 maps for each simulation length were combined and translated into FEMaps for each atom type. The resulting FEMaps for 10 ns simulations were used as a references for convergence study, and all other FEMaps at shorter simulation lengths were compared to those reference FEMaps.

Grid points within 5 Å of all ligands were selected and the free energy values on all grid points were compared by calculating the overlap coefficient (OC) (Equation 9).
Table 5: Fragment map type and its correspondence to co-solvent probe atoms

<table>
<thead>
<tr>
<th>Fragment map type</th>
<th>Co-solvent probe atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 General aromatic carbon</td>
<td>Carbon of benzene and its derivatives</td>
</tr>
<tr>
<td>2 Aromatic carbon, benzene</td>
<td>Carbon of benzene</td>
</tr>
<tr>
<td>3 Aromatic carbon, F-benzene</td>
<td>Carbon of F-benzene</td>
</tr>
<tr>
<td>4 Aromatic carbon, Cl-benzene</td>
<td>Carbon of Cl-benzene</td>
</tr>
<tr>
<td>5 Aromatic carbon, Br-benzene</td>
<td>Carbon of Br-benzene</td>
</tr>
<tr>
<td>6 Aromatic carbon, I-benzene</td>
<td>Carbon of I-benzene</td>
</tr>
<tr>
<td>7 Aromatic carbon, CH$_3$-benzene</td>
<td>Aromatic carbon of CH$_3$-benzene</td>
</tr>
<tr>
<td>8 Aromatic carbon, OH-benzene</td>
<td>Carbon of OH-benzene</td>
</tr>
<tr>
<td>9 Aromatic carbon, CF$_3$-benzene</td>
<td>Aromatic carbon of CF$_3$-benzene</td>
</tr>
<tr>
<td>10 Aromatic carbon, OCH$_3$-benzene</td>
<td>Aromatic carbon of OCH$_3$-benzene</td>
</tr>
<tr>
<td>11 Aromatic carbon, NH$_2$-benzene</td>
<td>Carbon of NH$_2$-benzene</td>
</tr>
<tr>
<td>12 Aliphatic carbon</td>
<td>Propane carbon</td>
</tr>
<tr>
<td>13 Carbon in CH$_3$</td>
<td>Aliphatic carbon of CH$_3$-benzene</td>
</tr>
<tr>
<td>14 Carbon in CF$_3$</td>
<td>Aliphatic carbon of CF$_3$-benzene</td>
</tr>
<tr>
<td>15 Carbon in OCH$_3$</td>
<td>Aliphatic carbon of OCH$_3$-benzene</td>
</tr>
<tr>
<td>16 Carbon in OCF$_3$</td>
<td>Aliphatic carbon of OCF$_3$-benzene</td>
</tr>
<tr>
<td>17 Nitrogen in amide</td>
<td>Nitrogen in formamide</td>
</tr>
<tr>
<td>18 Nitrogen in N$_2$-benzene</td>
<td>Carbon of NH$_2$-benzene</td>
</tr>
<tr>
<td>19 Carbonyl oxygen</td>
<td>Oxygen in acetaldehyde</td>
</tr>
<tr>
<td>20 Oxygen in amide</td>
<td>Oxygen in formamide</td>
</tr>
<tr>
<td>21 Oxygen, OCH$_3$-benzene</td>
<td>Oxygen in OCH$_3$-benzene</td>
</tr>
<tr>
<td>22 Oxygen, OCF$_3$-benzene</td>
<td>Oxygen in OCF$_3$-benzene</td>
</tr>
<tr>
<td>23 Fluorine</td>
<td>Fluorine of F-benzene</td>
</tr>
<tr>
<td>24 Fluorine, CF$_3$</td>
<td>Fluorine of CF$_3$-benzene</td>
</tr>
<tr>
<td>25 Chlorine</td>
<td>Chlorine of Cl-benzene</td>
</tr>
<tr>
<td>26 Bromine</td>
<td>Bromine of Br-benzene</td>
</tr>
<tr>
<td>27 Iodine</td>
<td>Iodine of I-benzene</td>
</tr>
</tbody>
</table>
\[ OC = \sum_{i=1}^{N} \min \left( \frac{Q_1^i}{\sum_{j=1}^{N} Q_1^j}; \frac{Q_2^i}{\sum_{j=1}^{N} Q_2^j} \right) \]  

(9)

\( Q_1^i \) and \( Q_2^i \) are the occupancy values of a specific atom type at grid point \( i \) in the set of short simulations and set of 10 ns long simulations, respectively. OC values range from 0 to 1 with the latter expressing full convergence/reproducibility between the two sets of simulations.

### 2.8 Comparison to Standard Co-Solvent Simulations

To validate the advantages to \( \lambda \)-dynamics co-solvent simulations over standard co-solvent simulations, we also performed standard MD simulations with the same set of co-solvent probes (Table 1) but without combining the various benzene derivatives into one probe group. In order to not increase the total number of co-solvent molecules, only the sixth fraction of co-solvent density was added for each benzene derivative, i.e. one sixth of 0.25 M. All other probes (propane, acetaldehyde and formamide) were added with a concentration of 0.25 M each.

### 2.9 Free Energy Predictions

We finally evaluated each ligand in our series using the FEMaps for free energy estimation. The free-energy contribution of each ligand atom was obtained from the values of the FEMap corresponding to the atom type and the atom location within the 3D grid. Summation over all atomistic free energy contributions provided the overall estimated free energy for each ligand configuration. The pose of each ligand was either obtained from the corresponding X-ray structure of the compound or by aligning the compound to the co-crystallized compounds based on their common scaffold. Pearson correlation coefficient \( r \) and Spearman rank correlation coefficient \( \rho \) were computed between predicted and experimental binding affinity.
3 Results and Discussion

3.1 hCatL

3.1.1 Inhibitor Halogen Atom Location Revealed by Fragment Maps

The FEMaps of all atom types in the binding site of hCatL are displayed in Figure 12. The highest occupancy regions of the aromatic carbon atom types (top two rows) displays significant overlap with the aromatic rings of the co-crystallized ligands. The most preferred regions for aliphatic carbon atoms (third row, left) co-localize partially with those of the aromatic carbon atom. Whereas the FEMap of hydrogen-bond acceptor and donor types (third line, right, and fourth line, left and middle) overlaps with the amide oxygen and nitrogen atoms of the ligand.

The FEMaps of the halogen atoms chlorine, bromine and iodine (bottom line) display high density in the S3 pocket in agreement with the observed halogen bond to Gly 61 (cf. Fig. 7). Additional peaks can be found in the S2 pocket which accompanies a chlorine atom in the ligand series, the S1 pocket and around the hydrophobic core of the ligand. This overlap is consistent with the hydrophobic character of the larger halogen atoms. The FEMap for fluorine in contrast does not display highly favorable density in the S3 pocket.

To validate the utility of the FEMaps unique to the λ-dynamics co-solvent approach, we focused our analysis on the S3 pocket where differences in substitution pattern on the benzene ring is the basis for the observed affinity differences in the ligand series studied here. Measuring the minimum free energy values of the FEMaps for the different atoms in this particular region displays excellent correlation with the observed structure-activity-relationships (Fig. 13). A Pearson’s $r$ value of 0.92 was observed when correlating the free energy gain or loss for the various modifications (F, CH$_3$, Cl, Br, I; cf. Fig. 7) to the minimum FEMap value in the S3 pocket for the corresponding atom type.

Comparison of the minimum FEMap values obtained from standard co-solvent simulations with the same set of probes lacks any correlation with the experimental structure-
Figure 12: Free energy density grids for different atom types obtained from $\lambda$-dynamics co-solvent simulation on hCatL. From top-left to bottom right: Aromatic carbon atoms in benzene at isolevel of 0.1 kcal/mol, in F-benzene (0.15 kcal/mol), in Cl-benzene (0.0 kcal/mol), in Br-benzene (-0.4 kcal/mol), in I-benzene (-0.4 kcal/mol), in methylbenzene (0.1 kcal/mol), aliphatic carbon of propane (-0.5 kcal/mol), methyl carbon of methylbenzene (-0.25 kcal/mol), nitrogen in amide (-1.0 kcal/mol), carbonyl oxygen (-1.5 kcal/mol), oxygen in amide (-1.0 kcal/mol), fluorine (0.0 kcal/mol), chlorine (-0.5 kcal/mol), bromine (-3.0 kcal/mol), and iodine (-3.0 kcal/mol).
Figure 13: Average free energy difference between substituted and unsubstituted ligand and its corresponding difference in minimum FEMap value in S3 pocket accommodating the modification. In blue the differences in minimum FEMap values from λ-co-solvent simulations shows significant correlation with observed differences in experimental free energies, whereas in orange no correlation was observed for the standard co-solvent simulations. For ligand pairs with the same modification on the para-position of the aryl group in the S3 pocket (e.g. H → Cl: IA1 → IA4, IB1 → IB3, and IC1 → IC3) the average free energy gain or loss was considered.

activity data (Fig. 13, orange; $r = -0.42$) highlighting the lack of convergence. This demonstrates the superior sampling power of λ-dynamics co-solvent simulations for a larger set of probes compared to standard co-solvent approaches. This comparison highlights also another advantage of λ-dynamics co-solvent simulations compared to standard co-solvent approaches, the feasibility to sample bulky groups in narrow subpockets. Whereas the standard co-solvent simulations have difficulty in sampling bulky probes such I-benzene, Br-benzene or CH$_3$-benzene in the subpocket near GLY 61 (highlighted by low atom type densities), λ-dynamics co-solvent simulations can access this subpocket by firstly approaching this location by less-bulky probes such as benzene or F-benzene, and secondly transforming into the larger probe molecules such as I-benzene or Br-benzene when the probe is interacting with GLY 61.
3.1.2 Convergence of Fragment FEMap

For each type of FEMap, we investigated how quickly convergence has been achieved in the \( \lambda \)-dynamics co-solvent simulations. FEMaps from trajectories of different lengths were quantitatively compared by calculating OCs (Figure 14).

Figure 14: Convergence of FEMaps of the various atom types represented by the probes used in current test system for \( \lambda \)-dynamics co-solvent simulations.

Good convergence (OC values above 0.85 and close to the maximum value of one) was obtained for all FEMaps for simulation lengths of 5 ns, with higher OC for the hydrophobic, aromatic, donor and acceptor maps. A slight reduction in OC values is expected for the atom types unique to the copied probes, i.e. methyl, fluorine, chlorine, bromide and iodine attached to benzene, as these atoms are not continuously turned on (\( \lambda < 0.85 \)). Whereas this reduction in OC values was expected in the context of \( \lambda \)-dynamics simulations, its magnitude remains small. This demonstrates the utility of \( \lambda \)-dynamics in co-solvent simulation settings to obtain converged sampling of a large variety of atom types.
Figure 14 also shows a plateauing off of OC values with increased simulation time for most atom types around 6-7 ns. Thus, a simulation setting with 50 instances of 7-8 ns length seem to be sufficient to obtain converged FEMaps when using \( \lambda \)-dynamics co-solvent simulations.

### 3.1.3 Correlation between Predicted and Experimental Binding Affinity

Figure 15 (top, left) shows the correlation between predicted free energy of each compounds based on the FEMaps and experimental free energy (see also Fig. 7). Significant correlation (Pearson’s correlation coefficient \( r=0.84 \)) and ranking (Spearman’s rank correlation coefficient \( \rho=0.85 \)) results were obtained, demonstrating the usefulness of the FEMaps obtained during \( \lambda \)-dynamics co-solvent simulations.

In comparison, no significant correlation (\( r=-0.13, \rho=-0.20 \)) was observed using the densities obtained from standard co-solvent simulations.

### 3.2 p53-Y220C

Similarly to hCatL, a strong correlation between the FEMaps for the halogen atom types co-localizing at the meta-aryl positions of the common scaffold (Figure 9) and the binding affinity was observed (Figure 16. Whereas the minimum FEMap value for chlorine around the Y position is -2.4 kcal/mol, the maximum values for bromine and iodine are -5.0 kcal/mol and -5.5 kcal/mol, respectively, correctly ranking compounds IB1, IB2 and IB3 (Figure 9). Also, the iodine FEMap around position X is more favorable as the corresponding chlorine FEMap, in agreement with the difference in affinity between IA1 and IB1. The overall predicted free energies using all FEMaps correlate well with the experimental affinities (\( r=0.98, \rho=1.00 \)), although we need to mention that the dataset for p53-Y220C is small (Figure 15, bottom left).
Figure 15: Correlation between predicted and experimental binding affinity using FEMaps from $\lambda$-dynamics co-solvent simulations for (top, left) hCatL, (top, right) HIV-1 protease, (bottom, left) p53, and (bottom, right) XIAP. Predicted free energies are scaled based on the linear correlation with experimental binding affinity (hCatL: $\Delta G_{\text{pred}} = 2.528 \cdot \Delta G_{\text{exp}} + 18.035, r = 0.84, \rho = 0.85$; HIV-1 protease: $\Delta G_{\text{pred}} = 0.5424 \cdot \Delta G_{\text{exp}} + 0.2603, r = 0.77, \rho = 0.84$; p53: $\Delta G_{\text{pred}} = 3.8636 \cdot \Delta G_{\text{exp}} + 8.7974, r = 0.98, \rho = 1.00$; XIAP: $\Delta G_{\text{pred}} = 0.6013 \cdot \Delta G_{\text{exp}} - 4.2265, r = 0.57, \rho = 0.71$).
Figure 16: Free energy density grids for different atom types obtained from $\lambda$-dynamics co-solvent simulation on p53-Y220C. From left to right: Chlorine (-2.0 kcal/mol), bromine (-4.0 kcal/mol), and iodine (-4.5 kcal/mol).

3.3 XIAP-BIR3

The observed SAR of the series of XIAP-BIR3 ligands depends strongly on the electronic properties of the indoline ring, with electron-poor rings being more potent than electron-rich rings. It was shown that the binding affinities correlate well with the Hammett substituent constants. Visualizing the electrostatic potential of the protein on the binding site surface (Figure 17, top-left) displays negative potential near the five-membered nitrogen-containing ring and positive potential towards the substitution (R group) on the benzene ring. The FEMaps of the different atom types are consistent with the observed SAR and the electrostatic potential map (Figure 17). For example, aromatic carbon atom types from electron deficient benzene rings (CF$_3$-benzene; Figure 17, top-right) display more favorable FEMaps in this region compared to carbon atom types of electron rich rings (NH$_2$-benzene; top-middle). Furthermore, partially negative substituents (e.g. CF$_3$; bottom-right) display a more favorable FEMap than positive substituents (e.g. CH$_3$; bottom-left), consistent with the observed SAR and underlining electrostatic interactions. Consequently, the FEMaps from the $\lambda$-dynamics co-solvent simulations are able to identify reasonable correlation between predicted and experimental binding affinities as observed in Figure 15, bottom-right ($r=0.57$, $p=0.71$).
Figure 17: Top-left: Electrostatic potential of protein on binding site surface. Free energy density grids for different atom types obtained from λ-dynamics co-solvent simulations on XIAP. From top-middle to bottom right: Aromatic carbon atoms in NH$_2$-benzene at isolevel of -0.1 kcal/mol, and in CF$_3$-benzene (-0.1 kcal/mol), methyl carbon of methylbenzene (0.3 kcal/mol), chlorine (-0.1 kcal/mol), and fluorine in CF$_3$ (-0.4 kcal/mol).

3.4 HIV-1 protease

Different to the three previous test systems, the SAR of the set of HIV-1 inhibitors depends on the substitution patterns on different ligand moieties, the two opposite terminal benzene groups. Furthermore, the substitutions are located at either meta or para positions of both rings. Despite this challenges, good correlation and ranking performance was observed using the FEMaps ($r=0.77$, $\rho=0.84$).

Analysis of the individual FEMaps (Figure 18) shows, for example, that the OCH$_3$-substitution on the para-position Y2 is preferred over the meta-position Y1: FEMaps of oxygen and carbon atoms in OCH$_3$-benzene probe (Figure 18, left column) have favorable contributions for the OCH$_3$-substitution on the para-position Y2 but not at the Y1 position, consistent with the SAR of IA1 versus IB1, and IA2 versus IB5. The FEMaps also reproduce the observed preference of OCH$_3$ (IB1-IB6) over OCF$_3$ substitution (ID1, ID2) (Figure 18, left versus middle column), a feature that could not have been modelled by the standard set.
of probe molecules typically used in previous co-solvent simulations.

The FEMaps are also able to perform similar predictions about the preferences of substitution on the other benzene ring (X1 vs X2). For example, the carbonyl oxygen of the acetate group in the X1 position coincides with favorable oxygen density of the carbonyl FEMap, but not when placed in X2 position, in agreement with the SAR (IB4 versus IB5, IC4 versus IC5).

Figure 18: From top-left to bottom right: Methyl carbon of OCH$_3$-benzene (-0.2 kcal/mol) and OCF$_3$-benzene (-0.2 kcal/mol), oxygen atom of OCH$_3$-benzene (-0.25 kcal/mol) and OCF$_3$-benzene (-0.25 kcal/mol), and carbonyl oxygen (-1.75 kcal/mol).

4 Conclusion

We presented the development of a novel co-solvent simulation method based on the $\lambda$-dynamics MD concept to allow for a diverse representation of functional groups at low co-solvent molarity to prevent aggregation of probes, in particular with hydrophobic character, and potential denaturation of the protein. The new method was validated on four different sets of ligands binding to hCatL, p53-Y220C, XIAP-BIR3 and HIV-1 protease. The density maps for the different atom types coincided with the ligands’ functional groups critical
for the observed interactions with the proteins. The free energy maps generated from the probe occupancies throughout the MD trajectories were correlating well with the observed differences in binding affinity for the ligand data sets. The theoretical basis for free energy calculation based on FEMaps assumes additivity of atomic free energy contributions, which is certainly a limiting factor of the method. Therefore, similar prediction accuracy compared to traditional free energy methods should not be expected. For clarity reasons, for each system, we only introduced one probe group with alternative probe copies in the current test studies. Our implementation, however, allows for the simulation of any number of probe groups with probe copies of similar chemical character. It should also be noted that the GPU-based implementation of $\lambda$-dynamics co-solvent simulation method allows for converged sampling of probe densities in less than a day on a workstation with two NVIDIA GPU 1080 Ti. With respect to the rich information content and simulation length, $\lambda$-dynamics co-solvent simulations may have the potential to become a routine method for structure-based drug design.

Acknowledgement

We acknowledge the Purdue Research Foundation for partially supporting this research.

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