Energetic Fingerprinting of Ligand Binding to Paralogous Proteins

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1 **Energetic Fingerprinting of Ligand Binding to Paralogous Proteins: The Case of the Apoptotic**
2 **Pathway**

3

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15

16 **Abstract**

17 Networks of biological molecules are key to cellular function, governing processes ranging from
18 signal cascade propagation to metabolic pathway regulation. Genetic duplication processes give rise to sets
19 of regulatory proteins that have evolved from a common ancestor, leading to interactomes whose
20 dysregulation is often associated with disease. A better understanding of the determinants of specificity at
21 interfaces shared by functionally related proteins is crucial to the rational design of novel
22 pharmacotherapeutic agents. To this end, a comprehensive dataset of drug and drug-like binders was
23 assembled for the Bcl-xL and Bcl-2 antiapoptotic proteins – archetypal examples of regulatory systems
24 governed by evolutionarily conserved protein-protein interactions. These were first used to derive a two-
25 dimensional quantitative structure-activity relationship (2D QSAR) model, predicting ligand specificity for
26 these homologous proteins. The strengths and weaknesses of high-throughput 2D QSAR were then
27 compared and contrasted to those of theoretically rigorous thermodynamic integration calculations
28 performed on 14 complexes of Bcl-xL-specific, Bcl-2-specific, and potent dual binders bound to the Bcl-xL
29 and Bcl-2 proteins. We demonstrate that free energy calculations provide an added layer of essential
30 information, which traditional QSAR cannot capture. Moreover, we show that protein energetic responses to
31 different ligands, expressed as per-residue energy values, can be used to fingerprint the protein-ligand
32 interaction, extending the framework of four-dimensional molecular dynamics/quantitative structure-activity
33 relationships (4D-MD/QSAR) towards the facilitation of future drug design strategies.

34 **Introduction**

35 Association between biopolymers is an essential part of physiological processes in all domains of
36 life. Deviations from the genetically predetermined innate interactome are often deleterious, manifesting
37 themselves in a broad spectrum of conditions, ranging from minor symptoms to debilitating syndromes.^{1,2}
38 For example, the antiapoptotic B-cell lymphoma-2 (Bcl-2) family of proteins, including Bcl-xL and Bcl-2,
39 bind to and inactivate BH3-domain proapoptotic proteins. Their overexpression overwhelms the cell's
40 proapoptotic defenses, facilitating malignant proliferation.³ Conversely, mutations that abrogate binding
41 between pro- and antiapoptotic proteins shift the cellular balance toward premature cell death, and give rise
42 to or are associated with degenerative diseases.^{4,5} Point mutations in leucine zipper transcription factors can
43 lead to altered dimerization and DNA binding, resulting in a great number of documented malignancies.⁶
44 Emulating regulatory protein – protein interactions with small molecule or (stapled) peptide analogues – the
45 so-called Bcl-inhibitors – has been successfully used to rectify imbalances in the apoptosis-regulation
46 pathway, and has produced numerous promising clinical candidates.⁷ Encouragingly, one Bcl-2-inhibitor –
47 Venetoclax (ABT-199) – has already obtained FDA-approval for cancer treatment.⁸ Targeting other
48 regulatory systems governed by inter- or intrafamily protein – protein recognition and association, such as
49 the ubiquitination pathway, two-component signal transduction, and G protein receptor regulation, is also
50 expected to provide future drug candidates for a wide range of conditions.⁹⁻¹¹ Thus, elucidating the intricacies
51 of protein – protein and protein – ligand association - the origins of specificity and affinity - is a long-
52 standing goal in the broader scientific field.

53 Computational strategies for modeling intermolecular interactions include those based on colloid
54 science,^{12,13} bioinformatics¹⁴⁻¹⁶ or force field-based approaches.^{17,18} Quantitative structure-activity
55 relationship (QSAR) studies are often employed to rationalize observed patterns in binding based on the
56 chemical moieties of the ligands that confer affinity and specificity,¹⁹⁻²¹ with more recent developments in
57 QSAR analysis seeing the explicit inclusion of structural data.²²⁻²⁴ Intrafamily protein – protein interactions
58 in key regulatory pathways, such as the Bcl-2 proteins, which regulate apoptosis,^{25,26} present a formidable
59 challenge to drug design and cancer therapy. The high similarity between family members, which have
60 arisen through gene duplication and subsequent divergence, makes off-target effects almost unavoidable and
61 limits the therapeutic efficacy of pharmacological agents. For example, the Bcl-2 protein has been targeted to

62 treat non-Hodgkin lymphoma and chronic lymphocytic leukaemia,²⁷ but undesired binding to the highly
63 similar Bcl-xL protein causes dose-limiting thrombocytopenia.²⁸ To date, all known peptide or small
64 molecule Bcl-2 binders also possess at least some residual affinity for Bcl-xL.²⁹⁻³³ Current data for Bcl-2
65 inhibitors in humans demonstrates that a selectivity of several thousand-fold for the designated target is
66 sufficient to make off-target binding clinically insignificant.^{8,34,35} The lower bound for selectivity, however,
67 has not been definitively established and may vary in different signaling pathways.

68 Medicinal chemists typically attempt to enhance binding affinity or achieve selectivity by optimizing
69 polar contacts and hydrogen bonds or salt bridges between a ligand and receptor under the assumption that
70 changes in ligands are small enough to not induce significant conformational change in the receptor. When
71 this is the case, it is straightforward to rationalize the origins of any enhancements to affinity afforded by
72 introducing hydrogen bond donors and acceptors or by enhanced van der Waals contacts. Even under these
73 favorable circumstances, however, it is not easy to anticipate every resulting change in binding energetics,
74 e.g. the free energy cost of reorganizing the receptor from the unbound to the bound ensemble or the change
75 in ligand conformational entropy upon binding.³⁶ Moreover, ligand – receptor interfaces often involve a great
76 number of interdependent interactions. Optimizing affinity and/or specificity becomes a complex,
77 multidimensional problem, and reducing the dimensionality of this problem is desirable. To this end, we
78 have compiled from literature^{7,8,24,30-33,35,37-51} a comprehensive dataset on binding affinity for Bcl-xL and Bcl-
79 2 inhibitors. We first present a two-dimensional quantitative structure-activity relationship (2D QSAR¹⁹⁻²¹)
80 analysis, based on low-level molecular descriptors, and compare that to an energetic analysis and 4D QSAR,
81 the fourth dimension being time, based on sampling of conformations²⁴ obtained from molecular dynamics
82 (MD) simulations for two dual Bcl-2/Bcl-xL binders, two Bcl-xL specific binders, and two Bcl-2-specific
83 binders from a congeneric series of compounds. 2D (low-level) QSAR results are compared and contrasted
84 with 4D (high-level) QSAR from thermodynamic integration calculations totaling 1.65 μ s, performed on 12
85 modeled protein – ligand complexes and 2 template protein – ligand complexes, obtained from the Protein
86 Data Bank (PDB) (Figure 1). We demonstrate how atomic-resolution MD simulations, coupled with free
87 energy calculations, can be used to more fully characterize protein – ligand complexes, and improve upon
88 traditional QSAR.

89

90 **Methods**

91 ***2D QSAR***

92 Only compounds shown to bind to the canonical groove of the antiapoptotic proteins were
93 considered for future analysis; these were converted to SMILES format. For each compound, 165 molecular
94 descriptors were calculated using RDKit.⁵³⁻⁶¹ Moreover, descriptors were additionally normalized by
95 molecular weight and heavy atom count; parameters, scaled to unit standard deviation, with zero or near zero
96 variance, were removed from consideration. Partial least squares regression was then performed on the
97 resulting set of descriptors, fitting the data to the logarithm of the affinity of a compound for Bcl-xL over
98 Bcl-2 ($\log_{10}(\text{affinity for Bcl-xL}/\text{affinity for Bcl-2})$) – a parameter henceforth referred to as specificity.
99 Ligands with a specificity below -1 are defined as Bcl-xL-selective, ligands with a specificity between -1 and
100 1 as dual binders, and ligands with a specificity greater than 1 as Bcl-2-specific. The final dataset consisted
101 of 57 Bcl-xL-specific compounds, 112 dual binders, and 19 Bcl-2-specific binders (Figure 2). The
102 compounds were sorted by increasing specificity and split into a training and test set for external validation⁶²
103 in a 3:1 ratio – for every three compounds in the training set, one was placed in the test set, moving from
104 Bcl-xL-selective to Bcl-2-selective ligands. The training dataset was used to derive a partial least squares
105 regression model, from which the specificity of the compounds in the test set was predicted. No compound
106 from the training set was present in the test set. To test the robustness and validity of the QSAR model,
107 Monte Carlo cross-validation was performed,^{63,64} i.e. a random number of random compounds in the test set
108 were swapped with compounds from the training set, after which the QSAR model was derived anew and
109 used to make predictions on the new test set, repeating this procedure 20,000 times. Only swaps within
110 classes were permitted, i.e. Bcl-xL selective compounds were exchanged only for Bcl-xL selective ligands,
111 Bcl-2-selective compounds were exchanged only for Bcl-2-selective ligands, and dual binders were
112 exchanged only for dual binders. Additionally, to remove any bias against the scarce Bcl-2 selective ligands,
113 the dataset was “normalized”, i.e. an analogous analysis was performed with only 19 compounds in each
114 class – 14 in the training set and 5 in the test set. Partial least squares regression was performed with the R
115 *pls* package.⁶⁵

116

117 ***System setup for template structure equilibration simulations***

118 Six of the compounds (see Figures 2 and 3) were modeled on the 2YXJ and 4LVT crystal structures
119 (ABT-737 (N3C) and ABT-263 (1XJ, Navitoclax) bound to Bcl-xL and Bcl-2, respectively); the models
120 were subjected to molecular dynamics simulation and free energy calculations.^{66,67} Before simulations of the
121 modeled complexes could be initiated, the template structures were simulated first; all simulations were
122 performed with the Amber14 suite.⁶⁸ N-termini were capped with an acetyl group, C-termini were capped
123 with a methylamino (-NHCH₃) group. Protein chains were protonated and solvated in a cubic box with
124 TIP3P water⁶⁹ with *tleap* with a minimal wall distance of 13 Å. 0.15 M NaCl was added to approximate a
125 physiological salt concentration whilst ensuring charge neutrality. Protonation states for the ligands were
126 assigned using ChemAxon's Calculator Plugins.^{70,71} Parameters for the ABT-737 and ABT-263 compounds
127 were obtained using the general Amber force field (GAFF 1.7⁷²) with AM1-BCC charges⁷³ using
128 *antechamber*.

129

130 *Simulation protocol for template structure equilibration simulations*

131 The solvated systems were subjected to a series of 2,000-steps energy minimization with a harmonic
132 restraint of 100 kcal mol⁻¹ Å² first applied to all heavy atoms, followed by all solute heavy atoms, followed
133 by protein heavy atoms. The systems were heated from 100 to 300 K over a period of 1 ns at constant
134 volume with 100 kcal mol⁻¹ Å² harmonic restraints on all heavy atoms, followed by constant pressure density
135 equilibration of 1 ns, followed by cooling to 100 K at constant volume with harmonic restraints. The protein-
136 ligand complexes were again subjected to 2,000 steps of energy minimization with restraints of 100 kcal mol⁻¹
137 Å² on protein heavy atoms, followed by 2,000 step minimization series with decreasing restraints of 50, 20,
138 and 10 kcal mol⁻¹ Å². The systems were then reheated from 100 to 300 K under constant volume conditions
139 over a period of 1 ns, with a harmonic restraint of 20 kcal mol⁻¹ Å² on protein and ligand heavy atoms,
140 followed by 1 ns of constant pressure density equilibration with restraints, followed by 1 ns of equilibration
141 with restraints on protein heavy atoms, followed by 1 ns of equilibration with 20 kcal mol⁻¹ Å² restraints on
142 Cα atoms only. The systems were then equilibrated for 1 ns without any restraints and simulated for 100 ns
143 under constant pressure (1 bar) and temperature (300 K) conditions, maintained with the Berendsen
144 barostat⁷⁴ and the Langevin thermostat.⁷⁵ Collision frequencies for temperature coupling were set to 2 ps⁻¹;
145 the pressure relaxation time was 2 ps. Both systems were simulated in four independent replicas with the

146 *ff14SB* force field. An 8.0 Å cutoff was used for van der Waals interactions. Long-range electrostatics were
147 computed with the particle-mesh Ewald (PME) scheme.⁷⁶ Bonds to hydrogen were constrained using the
148 SHAKE algorithm,⁷⁷ allowing for a 2 fs time step.

149

150 *System setup for thermodynamic integration simulations*

151 Trajectories were processed with *cpttraj* V14.25⁷⁸ to perform roto-translational alignment and
152 compute Cα and ligand heavy atom root-mean-square deviations (RMSDs). The instantaneous enthalpy of
153 binding (ΔH) between protein and ligand was also monitored every picosecond; ΔH was computed with
154 MMPBSA.py. Frames from the Bcl-xL – ABT-737 and Bcl-2 – ABT-263 trajectories were selected to serve
155 as templates for modelling the 12 antiapoptotic protein-ligand complexes and the subsequent free energy
156 calculations.⁷⁹ The six compounds from Figure 3 were modeled bound to Bcl-xL and Bcl-2 using
157 Schrodinger's Maestro suite.⁸⁰ Our guiding principle in modelling was to bury hydrophobic moieties in
158 hydrophobic pockets; polar fragments were anticipated to be solvent exposed. The placement of the
159 azaindoloxy moiety of compound 8 was guided by the positioning of an analogous indoloxy moiety in the
160 4MAN crystal structure of Bcl-2 bound to a similar compound,⁸ i.e. nearly parallel to the nitroaryl fragment.
161 Protonation states for the modeled ligands were assigned using ChemAxon's Calculator Plugins. Parameters
162 were obtained from the general Amber force field (GAFF 1.7) with AM1-BCC charges using *antechamber*.
163 The complexes were solvated in a cubic box with TIP4P water⁶⁹ and a minimal wall distance of 13 Å; NaCl
164 was added to a concentration of 0.15 M.

165

166 *Simulation protocol for thermodynamic integration simulations*

167 One-step thermodynamic integration with softcore potentials and linear scaling^{81,82} was performed on
168 the ABT-737 and ABT-263 compounds bound to Bcl-xL and Bcl-2, transforming each of them into the six
169 target compounds (Figure 3) bound to the two proteins, as well as isolated in solution. Henceforth, this is
170 termed the “forward” transformation, as opposed to the reverse process of transforming the model ligand into
171 the template molecule, which was also performed. Moreover, the absolute free energies of binding of the
172 template ligands⁸³ were computed by decoupling them from the computational box⁸⁴ (for the reverse

173 transformation) and by inserting them into the computational box (for the forward transformation).

174 The solvated model complexes were subjected to 2,000 steps of energy minimization with a
175 harmonic restraint of 20 kcal mol⁻¹ Å² on protein and ligand heavy atoms. The structures were heated from
176 100 to 300 K under constant volume conditions for 10 ps with 20 kcal mol⁻¹ Å² restraints on protein and
177 ligand heavy atoms, followed by 10 ps of restrained density equilibration, and 2.5 ns of unrestrained constant
178 pressure dynamics in each λ -window. The time step was set to 1 fs; bonds to hydrogen were not constrained
179 during thermodynamic integration simulations. Transformations were carried out with 11 λ -windows for the
180 forward ($\lambda = 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0$) and reverse ($\lambda = 0.005, 0.1, 0.2, 0.3, 0.4, 0.5,$
181 $0.6, 0.7, 0.8, 0.9, 0.995$) processes and default settings for the *scalpha* (0.5) and *scbeta* (12 Å²) parameters,
182 which control the softness of the potential. Energy minimization, heating, density equilibration, and
183 production dynamics were all performed with potential energy functions, corresponding to the λ -value of
184 every λ -window, thus avoiding any Hamiltonian lag.⁸⁵ Forward, i.e. template-to-model, transformations were
185 carried out with the *pmemd* module of Amber, whereas reverse (model-to-template) transformations were
186 performed with *sander*. All transformations were carried out with the *ff14SB* force field in three independent
187 replicas under NPT conditions.

188

189 ***Free energy analysis***

190 Autocorrelation times (τ) for the free energy calculations were computed from the derivative of the
191 potential with respect to λ ($\delta V/\delta \lambda$). The pressure-volume component of the free energy was considered
192 marginal and ignored, as is typically the case in biomolecular simulations.⁸⁶⁻⁸⁹ Moreover, for the *sander*
193 transformations, per-residue energy decomposition was enabled, adding 1–4 energy terms to internal energy
194 terms. Per-residue, i.e. residue-by-residue, decomposition provides a quantitative assessment of each
195 individual residue's energetic contribution to the binding process – favorable, unfavorable, or neutral.
196 Energy values for the protein residues from the last 1 ns of simulation in each λ -window were saved,
197 whereas water and ions were disregarded in subsequent analysis. Per-residue energy values were compared
198 for the model and template ligands bound to Bcl-xL and Bcl-2 for the three replicas by computing Pearson
199 correlation coefficients. When comparing ligands bound to the same protein, Pearson correlations from all

200 protein residues were computed. When comparing ligands across proteins, values for residues 25 – 137 for
201 Bcl-xL and 27 – 139 for Bcl-2 were used. Helix 1, which is not involved in binding, was discarded, i.e.
202 comparisons were only made between the core fold residues among the two proteins, which have a 1:1
203 correspondence in sequence and structure. Correlation plots were generated with the R *corrplot* package.^{90,91}
204 Tanimoto similarities between the eight ligands from Figure 3, topological fingerprint similarities, and
205 MACCS key similarities were computed with RDKit. MACCS keys are constructed via a molecular
206 perception algorithm,⁹² which uses atom typing and molecular context to translate ligand structures into bit
207 vectors, where the presence of a particular property, e.g. a para-substituent, is indicated with a 1 in a
208 specified position in the vector and its absence is indicated with a 0; vector similarity, then, corresponds to
209 ligand similarity, which can thus be quantified using the difference between the bit vectors. Tanimoto
210 coefficients and topological fingerprints are related measures of similarity, also based on bit vectors of
211 molecular properties and connectivity.⁵⁷ Tanimoto, topological, and MACCS key similarities were then
212 compared to per-residue energy patterns, obtained from free energy analysis.

213

214 **4D QSAR**

215 In addition to the 2D QSAR analysis performed on a large set of compounds, we performed 4D
216 QSAR on compounds 3, 4, 5, 6, 7, and 8 (see Figures 2 and 3), using the per-residue energy values, obtained
217 from the thermodynamic integration calculations. Analogously to the 2D QSAR analysis, the descriptors,
218 which in this case are the energy values, were scaled to unit variance, and used to perform partial least
219 squares regression, fitting the data to the specificity. Training was performed on compounds 3, 5, and 7,
220 testing was performed on compounds 4, 6, and 8 (Venetoclax, ABT-199), and vice versa. To verify the
221 robustness of our results, for each case, 20,000 random combinations of energy values from replicas 1, 2, and
222 3 for each compound in the test and training set were used. Partial least squares regression was performed
223 using energy values for residues 25 – 137 for Bcl-xL and residues 27 – 139 for Bcl-2.

224

225 **Results**

226 **2D QSAR**

227 The initial QSAR model, derived from the entire training set, performed reasonably well in the
228 external validation on the 47-compound test set, with an overall R^2 between predicted and measured
229 specificity of 0.48 (Figure 4 A). Most dual binders were predicted accurately, as were most Bcl-xL-selective
230 compounds. Compounds A, B, C, and D, which belong to different chemical series, lie close to the $x = y$
231 identity line (see Figure 2 and 4 B), as do compounds 3, 4, N3C (ABT-737), and 1XJ (ABT-263), which
232 belong to the arylsulphoneamidoaryl series, which dominates the set. Arylsulphoneamidoaryl compounds 5
233 and 6 have their specificity slightly shifted up towards the dual binders, likely because
234 arylsulphoneamidoaryl dual binders dominate the set. Compounds E, F, and G – a set of
235 tetrahydroisoquinoline derivatives – are well predicted, as similar compounds are present in the training set.
236 Only the Bcl-2-selective arylsulphoneamidoaryls are mispredicted by more than 3 units of specificity,
237 eroding overall R^2 and reducing the slope of the line of best fit for the predictions. Randomizing the training
238 and test sets 20,000 times produced similar results, with R^2 values varying around 0.5. The standard
239 deviations of predicted specificities for most compounds were around 0.5 with the notable exception of the
240 Bcl-2-specific binders, where predicted specificities varied by up to 2 log units. Large decreases in predictive
241 performance were mostly associated with ligand randomizations where training was performed on
242 compounds from one chemical series and used to perform predictions on a very different series of
243 compounds. Performing the analysis with reduced training and test sets (Figure 4 C) improves the prediction
244 accuracy for compound B, which belongs to a fairly sparsely represented chemical series. Again, compounds
245 E, F, and G are accurately predicted, as compounds from this series are present in the training set. There is a
246 slight downshift in predicted specificities for arylsulphoneamidoaryls, as evidenced by compounds 3, 4, 5, 6,
247 7, 8, 1XJ, and N3C, likely because dual arylsulphoneamidoaryl binders do not dominate the “normalized”
248 data set. Moreover, fluctuations in R^2 become more pronounced, as one or two severely mispredicted
249 compounds influence R^2 much more dramatically with the reduced test set. Again, only large, Bcl-2-specific
250 arylsulphoneamidoaryls were severely mispredicted. Indeed, removing the two Bcl-2-selective
251 arylsulphoneamidoaryls from the test sets improved R^2 of the initial models to 0.70 and 0.90 for the 45 and
252 13 compound sets, respectively.

253

254 *Template structure simulations*

100-ns MD equilibration simulations were performed in quadruplicate on the template compounds bound to Bcl-xL and Bcl-2, in order to relieve any strain potentially present in the complexes and to assess the stability of the structures (Figure 5). The proteins were stable over the course of the 100-ns trajectories, with C α RMSDs fluctuating around a steady value below 1.5 Å. The ABT-263 ligand remained highly stable throughout all four replicas, whereas ABT-737 appears to have a greater mobility in the hydrophobic groove of the Bcl-xL protein. We prioritized proximity to the crystal structure conformations of the ligands, followed by low C α RMSD values for the proteins. MM-PBSA energies were also monitored to further assess the energetics of binding; these indicated stable binding throughout the trajectories. Suitable snapshots from the simulations (see Figure 5) were chosen to serve as templates for modeling the different protein-ligand complexes. These models were subsequently subjected to thermodynamic integration calculations.

Alchemical transformations

We next performed thermodynamic integration calculations^{82,93} on the ABT-737 and ABT-263 compounds, bound to Bcl-xL and Bcl-2, transforming each of them into the six compounds in Figure 3 bound to the two proteins, in triplicate. We also performed the reverse calculations, again in triplicate. Thus, relative binding affinities ($\Delta\Delta G$ s) were calculated. Moreover, we also decoupled/inserted the template ligands from/into the binding pocket and free in solution to compute their absolute binding affinities (ΔG s). Autocorrelation times varied from several picoseconds to several hundred picoseconds among the different λ -windows. Thus, performing long enough simulations ($> 50\tau$) to obtain a sufficient number of truly uncorrelated samples in all λ -windows was unfeasible. We, therefore, present ΔG s and convergence plots from $\delta V/\delta\lambda$ values taken every picosecond; these differ marginally from results sampled 10 ps apart. The means of the free energies for the transformations and their standard deviations are shown in Figure 6; the corresponding convergence and C α RMSD plots are presented in Figures S1 – S8. The proteins were stable throughout the 2.5 ns of dynamics in each λ -window, with RMSDs typically remaining below 1 Å. For most of the ligands, the results from the forward and backward transformations are in good agreement, being of similar magnitude but opposite sign (Figure 6). This indicates that we have attained sufficient sampling, and confirms the equivalence of the *sander* and *pmemd* molecular dynamics engines in terms of output.⁸⁹ In most

replicas, for the relative transformations, the $\Delta\Delta G$ converged to a value near the experimental $\Delta\Delta G$ over the course of the simulations, in certain cases within “chemical accuracy,” i.e. within 1 kcal mol⁻¹ (see Figures S1, S2, S5 and S6). Only for the transformations where the ligands were inserted into or decoupled from the computational box were computed ΔG values off from experimental ones by a greater margin, in certain cases by more than 10 kcal mol⁻¹. Moreover, with the exception of ligand 8 in isolation, these were the only simulations with large differences in computed ΔG s among replicas (standard deviations > 5 kcal mol⁻¹, see Figure 6). The standard deviations of computed (Δ) ΔG values among replicas are several-fold larger than (Δ) ΔG errors within replicas. The standard deviations, therefore, are the values we present (Figure 6) – most are around 1 kcal/mol.

291

292 ***Energetic fingerprinting***

We next compared the protein per-residue energies from the *sander* simulations when the two proteins are bound to different compounds, as well as between Bcl-xL and Bcl-2 when bound to the same compound. To facilitate comparison, for each compound in each of the energetic similarity panels (Figure 7), we prepend the number of the compound (3, 4, 5, 6, 7, and 8) with N3 or 1X to designate which protein the examined compound is bound to (the N3 and 1X prefixes derive from the N3C and 1XJ template ligands bound to Bcl-xL and Bcl-2, respectively). Examining the correlation plots of the Bcl-xL per-residue energies when the protein is bound to different ligands (Figure 7 A) reveals that the strong dual binders – compounds 3 and 4 – are energetically highly similar to each other and to the Bcl-xL selective compounds – 5 and 6 – which are also potent Bcl-xL binders. The pattern becomes intuitive by noticing the large squares in the energetic similarity panels, ranging from compound 3 (labeled as N33 in the energy panels) to compound 6 (labeled as N36). Conversely, the dual and Bcl-xL-selective ligands are energetically distinct from the Bcl-2-specific compounds, which are highly similar to each other (small squares in the bottom left of the panels). Furthermore, compound 7, which is the weakest Bcl-xL binder in the entire set, has the least energetic similarity to any of the other remaining compounds in two of the three replicas. This appears to be the general pattern for the Bcl-2 complexes as well (Figure 7 B), with results being highly consistent among the three replicas. Moreover, if we compare energetic patterns in between proteins, i.e. correlate the energies of residues 25 – 137 of Bcl-xL with 27 – 139 of Bcl-2, it becomes evident that the dual binders elicit similar

energetic patterns in the two proteins (see the highlighted diagonals in Figure 8 A). Conversely, the Bcl-xL-specific binders appear to evoke less similar energetic responses, whereas the Bcl-2-selective compounds, like the dual binders, also elicit similar responses in Bcl-xL and Bcl-2. If we compare energetic similarities to Tanimoto, topological, or MACCS key similarities – typical metrics of compound likeness in chemoinformatics – an interesting pattern emerges – dual binders (compounds 3 and 4) are structurally and energetically similar, Bcl-xL-selective binders (5 and 6) are structurally similar, but energetically distinct, whereas Bcl-2-selective ligands (7 and 8) are structurally different, but energetically similar (compare the three squares lying on the highlighted diagonals in Figure 8 A). Finally, Bcl-xL-selective compounds are energetically least similar to Bcl-2-selective compounds than to any other compound in the dataset (see the off-diagonal squares in Figure 8 A).

320

4D QSAR

Training on per-residue energy values from MD simulations to predict ligand specificity demonstrates that the Bcl-2-selective ligands are predicted within 2 log units of experimental values; dual binders lie within or close to the square, delimited by the [-1,1] range; and Bcl-xL-specific binders are either well predicted or have slightly upshifted specificities, depending on the choice of test and training sets (Figure 9). Moreover, results are consistent over the two 20,000-interreplica randomizations, with mostly small standard deviations of predicted specificities in the range ~0.1 – 0.5.

328

Discussion

The antiapoptotic proteins remained stable throughout the 100-ns trajectories, in agreement with extensive previous simulations.¹⁷ The ABT-263 template compound remained stably bound to the Bcl-2 protein, whereas the highly similar ABT-737 template displayed greater mobility, particularly the fragment that fits into pocket 2 of the protein. This appears to be due to the topology of the Bcl-xL groove around the biphenyl fragment of ABT-737, which is shallower than that of Bcl-2, in part due to the A104D and S122R amino acid substitutions (nomenclature is Bcl-xL → Bcl-2; numbering corresponds to the canonical Bcl-xL sequence). Pocket 2 (labeled “p2” in Figure 1) accommodates the biphenyl fragment of ABT-737 and its

337 corresponding moieties in other ligands. Loss of this interaction, in turn, facilitates the loss of the
338 intramolecular π -stacking interaction between the thiophenyl and nitrophenyl moieties in pocket 4 (labeled
339 “p4” in Figure 1) – a characteristic feature of the thiophenyl-bearing sulphoneamidoaryl class of Bcl-
340 inhibitors, observed in multiple X-ray and NMR structures.^{8,31,49,94-96} Given the relative shallowness of the
341 Bcl-xL rim around p2 and the greater mobility of the template compound bound to Bcl-xL, observed in our
342 simulations, we expect compounds of similar affinity for Bcl-xL and Bcl-2 to display greater k_{off} rates for
343 Bcl-xL than Bcl-2.

344 We have presented thermodynamic integration free energy calculations^{88,98} on large, complex drug
345 and drug-like molecules. We find it reassuring that most of the relative transformations approach a value
346 within ~ 2 kcal mol⁻¹ from the experimental $\Delta\Delta G$ (see Figure 6 B and the free energy convergence plots in
347 Figures S1, S2, S5 and S6). As may be expected, only the absolute ΔG calculations are off by a greater
348 margin, sometimes more than 10 kcal mol⁻¹. In part, this likely arises from force field inaccuracies. Added to
349 this, insertion or deletion of the ligand is a much greater transformation than transforming the template
350 compounds into the models or vice versa, and thus necessitates more sampling to alleviate any hysteresis.
351 Finally, the alchemical transformations we have performed involve charged species. This introduces an error
352 of a complex nature, involving periodicity-induced net-charge interactions, periodicity-induced net-charge
353 under-solvation, discrete solvent effects, and residual integrated potential effects.¹⁰⁴ This error is likely to be
354 compounded by the circumstance that we are transforming the template ligands N3C and 1XJ, which have a
355 net charge of +2, into the model ligands, which all have a net charge of +1, or vice versa. As all template and
356 model ligands have a positive net charge, the error stemming from ligand charge is likely to largely cancel in
357 the relative transformations, and judging by the respective free energy convergence plots (see Figures S1, S2,
358 S5 and S6) it does. However, in the absolute free energy calculations, the transformed state has a charge of 0
359 (when the template ligand is decoupled from the simulation box) or +2 (when it is inserted into the
360 simulation box), i.e. the change in charge in the template compound simulations (+2 to 0 or 0 to +2) is
361 different from the change in charge in the relative transformations (+2 to +1 or +1 to +2), which diminishes
362 error cancelation in the template simulations. Indeed, the $\Delta\Delta G$ curves converge to a value that is close to the
363 experimental value (the purple horizontal lines in Figures S1, S2, S5 and S6) for compounds 3, 4, 5, 6, 7, and
364 8, but are off by a greater margin for the template compounds (N3C and 1XJ). Moreover, since all of the

365 template-to-model (and vice versa) transformations involve the same change in ligand charge (+2 to +1 and
366 +1 to +2, respectively), one can confidently compare these transformations among each other. In other
367 words, for any two of the model compounds, in a situation where experimental affinity measurements are not
368 available, for example, one would compare the calculated $\Delta\Delta G$ s (as they are a measure of predicted affinity)
369 and subtract one $\Delta\Delta G$ from the other to discern which compound has the greater affinity for the target
370 protein. As all template-to-model and model-to-template transformations contain largely the same error
371 stemming from the change in ligand charge, it will mostly cancel during the subtraction, i.e. comparison.
372 However, the template compound simulations, where the ligand is decoupled or inserted from/into the
373 simulation box, involve a different charge transformation (+2 to 0 or 0 to +2), making it more difficult to
374 compare them to the rest of the simulations. This is a well established shortcoming of MD – small
375 perturbations can be assessed more reliably than large ones, relative energies can be computed much more
376 accurately than absolute ones. In this work, we have shown that large and complex drug molecules can be
377 reliably parameterized with a general force field, such as GAFF, and a rapid method for charge estimation,
378 such as AM1-BCC. Absolute binding energy calculations of charged species, however, necessitate further
379 theoretical and methodological developments.^{36,104-106}

380 The foregoing error also influences the per-residue values obtained from energy decomposition. To
381 assess its influence, we also performed simulations on compounds 5 and 6 in the +2 protonation state (see
382 Figure 8 B). This state is less likely because the piperazinyl nitrogen is bound to electron withdrawing
383 groups. Moreover, protonation of the nitrogen atom stabilizes it in a tetrahedral geometry, which facilitated
384 inversions in the terminal moieties where the phenyl groups pointed outward into the solvent and the polar
385 atoms pointed inward into the hydrophobic groove. Such an arrangement seems unlikely. Conversely, the +1
386 protonation state stabilized the ligands in the groove. In the case of Bcl-xL, coupled with the relative
387 shallowness of the groove around pocket 2, it also increased the propensity of the terminal moieties of
388 ligands 5 and 6 to adopt an extended conformation along the surface of the groove. Such an arrangement has
389 been observed crystallographically in similar compounds bound to Bcl-xL.³¹ Nevertheless, simulating
390 compounds 5 and 6 in the +2 state reveals an interesting aspect of free energy calculations. It appears that the
391 charging/decharging error strongly influences the per-residue values. These are the only simulations where
392 the ligand net charge does not change during the alchemical transformation. The per-residue values are quite

different from the rest of the simulations, where one of the ligand end states has a charge of +2 or 0, the other +1 or 0 (0 when the ligand is decoupled from the system ($\lambda = 1$) or inserted into the system ($\lambda = 0$)); this is the only instance where anti-correlations appear (Figure 8 B). Since the error resulting from the change in charge is present in all other transformations, we expect the energetic patterns reported herein to be *bona fide*, particularly given the reproducibility across three independent replicas.

QSAR models derived from the arylsulfonamidearyl series of compounds performed well when predicting properties of other arylsulfonamidearyls. Similarly, training on the tetrahydroisoquinolines produced predictive models for other tetrahydroisoquinolines. However, training on one series of compounds to predict the properties of another unsurprisingly leads to poor results. Moreover, Bcl-2-specific arylsulfonamidearyls, of which there are only two in our entire dataset, are poorly predicted by the present models. This is a well-known shortcoming of traditional QSAR models – they are much better at interpolation than extrapolation^{107,108} – and even the most sophisticated QSAR models may perform poorly on challenging targets.¹⁰⁹ Moreover, predictive performance is sensitive to the specific choice of test and training compounds, very much so in the case of small datasets. Quite often, QSAR models suffer from “activity cliffs”¹¹⁰ where a small change in a compound, such as introduction or removal of a hydroxyl or a methyl group, translates into a large change in activity. Most models, based on 2D descriptors, or 2D measures of similarity, such as Tanimoto coefficients or MACCS keys, would experience little change upon such an alteration in a large molecule. It appears that two-dimensional models and descriptors simply do not contain in themselves all the information relevant to ligand activity. Similar findings have been reported previously.^{111,112} In hindsight, this is not surprising – the descriptors used to construct low-dimensional QSAR models are, by the very nature of the technique, invariant with respect to the macromolecule – a major determinant of the binding process; they are also invariant with respect to time. Moreover, most 0-, 1-, and 2-dimensional descriptors do not discriminate between enantiomers. In stark contrast, free energy calculations and energy decomposition should, at least in principle, provide a detailed understanding of the binding process. Distinguishing enantiomers, for example, becomes trivial by the nature of the technique – where one enantiomer makes favorable interactions, and the other does not, there will exist a difference in potentials between the two systems, a difference that will manifest itself in a computationally detectable $\Delta\Delta G$. Moreover, the difference in residue – ligand contacts should, again at least in principle, be evident on a

per-residue level; finer-grained decomposition is also possible.¹¹³

Our work corroborates this line of reasoning. Despite the lower training:test set ratio in the 4D versus the 2D QSAR analysis (1:1 vs 3:1, respectively), the 4D models performed considerably better at estimating the activities for the challenging Bcl-2-selective binders, outperforming 2D models by around 1 log unit of specificity, with 2- to 10-fold lower standard deviations among the 20,000 combinations. Furthermore, while an analogous analysis on the same set of compounds using 2D descriptors exhibits a comparable performance in terms of absolute error (compare Figure 9 and S9), crucially, it again mispredicts the Bcl-2-selective compounds as dual binders. These results indicate that per-residue energies are a much more suitable, albeit more expensive, descriptor of activity. Indeed, 2D QSAR builds predictive models based on properties such as molecular weight, net charge, logP, the number of aliphatic or aromatic carbons in a compound, and molecular connectivity or branching⁵⁷ – typically some linear or nonlinear combination thereof. However, these descriptors are too degenerate and too coarse to capture the fine difference in binding between paralogs. In stark contrast, molecular dynamics and free energy calculations provide a detailed, atomistic level of description of binding and energetics. It would then seem likely that the per-residue energies are a more fine-grained descriptor, which affords a higher-resolution account of the binding process. Indeed, atomistic simulation and decomposition analysis have already been successfully employed to unravel the complex binding patterns in the Bcl-2-family, explaining why certain protein – peptide pairs bind with high affinity, whereas others do not.¹⁷ Moreover, in contrast to low-level QSAR, properties computed from simulations are time-dependent. This is another key asset of MD, as monitoring RMSDs and free energies over time allows detecting the point of convergence in simulations of off-equilibrium models, generated in the absence of an X-ray or NMR structure.

We also note the remarkable accuracy of predicting specificities using the +2 protonation state for ligands 5 and 6 (Figure S10). Thus, despite the considerations we have discussed previously, we cannot completely rule out other factors perturbing the pK_a 's of the ligands, stabilizing the +2 state. In such a scenario, the energetic pattern previously discussed still holds – the dual binders (3 and 4) are structurally and energetically similar; Bcl-xL-selective binders (5 and 6) are structurally similar, but energetically distinct; and, finally, Bcl-2-selective ligands (7 and 8) are structurally different, but energetically similar (see the correlation coefficients in Figure 8 B). In the +2 state, compounds 5 and 6 have a unique energetic

449 fingerprint, setting them apart from each other and the rest of the ligands, making specificity predictions
450 using per-residue energies highly accurate – compounds 3, 4, 5, and 6 lie almost ideally on the identity line,
451 with only 7 and 8 deviating more substantially from it – likely a consequence of the disparity in affinity for
452 Bcl-2 between the training and test compound for the Bcl-2-selectives.

453 With Figure 9 and Figures S9 – S10, we provide a direct and fair comparison between the
454 capabilities of 2D descriptors and models with 4D ones on the same set of compounds. While R^2 values from
455 just a few data points are not informative in themselves, the key conclusion to be drawn from these plots and
456 the plots in Figure 4 is that 2D models cannot correctly predict the selectivity of the Bcl-2-specific
457 compounds, whereas 4D models are quite successful – they reproduce the correct trend in selectivity across
458 the entire spectrum of compounds, with rather small quantitative errors for the Bcl-2-selectives. It is also
459 instructive to consider the applicability domain of the models we present. One simple way of estimating the
460 applicability domain is to use the standard deviation of predictions from multiple models²⁰ (in our case - the
461 20,000 randomizations) - small variations indicate that the models are robust and being used within the
462 boundaries of their applicability domain; large variations suggest otherwise. Using this metric, it becomes
463 apparent that the applicability domain of the 2D QSAR models spans from the Bcl-xL-selective compounds
464 up to and including the dual binders; it starts from the lower regions of the specificity axis and ends in the
465 region around +1. Conversely, 4D models extend the applicability domain beyond +1, well into Bcl-2-
466 specific territory. Indeed, comparison between the predicted and experimental values confirms this
467 reasoning.

468 Other studies have also demonstrated that QSAR models based on ligand properties computed from
469 MD trajectories can help to better predict ligand biological activity and resolve activity cliffs compared to
470 2D- and 3D-based models.¹¹⁴ What is particularly noteworthy about the study by Ash and Fourches on a set
471 of kinase inhibitors¹¹⁴ is that for some of the descriptors, it is not the means of the descriptors that correlate
472 with biological activity, but their standard deviations over the course of the simulations, with more active
473 ligands displaying greater variance. The authors hypothesize that this is a result of the ligands adapting to the
474 dynamic kinase pocket, and demonstrate how 4D QSAR models add an extra layer of information content
475 over 3D models. Perhaps most important of all, they show that 4D models are capable of yielding
476 information and features that no lower-level model is capable of providing. Furthermore, dynamics-based

477 pipelines such as *ensemble* Comparative Residue-Interaction Analysis (*e*CORIA¹¹⁵) allow one to quantify the
478 imperative properties and interactions in the binding process, which enhances the interpretability of modern
479 QSAR models – an increasing requirement in legislation regulating the development of pharmaceuticals,
480 agrochemicals, and cosmetics.

481 While characterizing a ligand's dynamic behavior in complex with a biological macromolecule is a
482 prerequisite to fully understanding binding, understanding the macromolecule's behavior is also essential. In
483 the case of Bcl-2-selective arylsulfonamidearyls, for instance, traditional QSAR models would perform
484 poorly when predicting their specificity, as most presently available arylsulfonamidearyls are dual binders or
485 Bcl-xL selective. MD simulations and free energy calculations, however, demonstrate that the Bcl-xL and
486 Bcl-2 proteins differentiate between these ligands from dual and Bcl-xL selective binders, as evident from
487 our current analysis, which can be viewed as an assessment of a protein's energetic response to a particular
488 binding partner. Including the water molecules and ions in the per-residue analysis in such calculations
489 would enable detecting bridging¹¹⁶ or trapped¹¹⁷ waters/ions, which are often important, but neglected,
490 factors in the binding process. This may mitigate the need for GIST-¹¹⁸ or 3D-RISM-type calculations,¹¹⁹ and
491 reduce the human workload, for example in a lead-optimization campaign, at no additional computational
492 cost.

494 **Conclusions**

495 A means of characterizing protein – ligand/peptide/protein interactions is presented and used to
496 demonstrate how macromolecules energetically distinguish between different binding partners. This work
497 paves the way for developing and validating more predictive models and descriptors, based on per residue
498 energies. Such energy-based four-dimensional descriptors^{120,121} would constitute a composite of multiple
499 lower-level descriptors, encompassing in themselves aspects of the binding process such as steric and atom-
500 type propensities for given ligands at given receptor locations, e.g. polar groups with positive partial charges
501 in the vicinity of a key hot spot or specificity determinant.^{17,122} In the short-to-medium term, 4D QSAR and
502 MD remain less accessible than 2D QSAR, as the former is several orders of magnitude more expensive. 2D
503 QSAR requires seconds to minutes to perform on any personal computer, whereas simulations such as those
504 reported here require several weeks of compute time on a large high-performance cluster. Furthermore, the

pre- and post-processing of thermodynamic integration simulations are labor intensive tasks that require specialist knowledge. However, even at the present time, MD simulations and 4D QSAR have considerable merits, which outweigh their greater expense. For example, they can steer hit-to-lead and lead-optimization campaigns in an academic or industrial setting in a way that 2D QSAR cannot. We have shown that MD-based QSAR outperforms 2D QSAR in predicting ligand selectivity, despite the lower training:test set ratio. This holds not only in quantitative (absolute error and R^2) but, more importantly, in qualitative terms – QSAR models based on low-level descriptors cannot predict the selectivity of compounds 7 and 8 for Bcl-2, whereas energy-based models are quite successful at predicting specificity over the entire spectrum – from Bcl-xL-selectives, through dual binders, to Bcl-2-specific molecules (compare Figures 4, 9, S9, and S10). Indeed, MD- and ensemble-based pipelines extend the applicability domain of models well beyond what 2D QSAR can provide. Remarkably, our 4D models correctly predict the selectivity of compound 8. This is notable because compound 8 (Venetoclax, ABT-199) is a highly challenging target for traditional 2D QSAR – it is the most Bcl-2-selective compound known to date. Given the constant ongoing improvements in force fields,¹²³ automation of free energy calculations and construction of optimal thermodynamic cycles,^{79,124} and the increase in computing power at an ever decreasing cost, it is to be expected that higher-dimensional descriptors and models will become more prevalent, at the expense of lower-level descriptors and models.

521

522 **Supporting Information.**

Free-energy convergence and RMSDs for alchemical calculations, and supplementary 2D/4D QSAR data (PDF).

525

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536

537 **Conflict of interest**

538 The authors declare no conflict of interest.

539

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547 **References**

- 548 1. Gonzalez, M.W.; Kann, M.G. Chapter 4: Protein Interactions and Disease. *PLoS Comput. Biol.* **2012**,
549 8, e1002819.
- 550 2. Rodriguez-Soca, Y.; Munteanu, C.R.; Dorado, J.; Pazos, A.; Prado-Prado, FJ.; González-Díaz, H.
551 Trypano-PPI: a Web Server for Prediction of Unique Targets in Trypanosome Proteome by Using
552 Electrostatic Parameters of Protein-Protein Interactions. *J. Proteome. Res.* **2010**, 9, 1182–1190.
- 553 3. Park, D.; Magis, A.T.; Li, R.; Owonikoko, T.K.; Sica, G.L.; Sun, S.Y.; Ramalingam, S.S.; Khuri,
554 F.R.; Curran, W.J.; Deng, X. Novel Small-Molecule Inhibitors of Bcl-XL to Treat Lung Cancer. *Cancer Res.*
555 **2013**, 73, 5485–5496.
- 556 4. Bouillet, P.; Cory, S.; Zhang, L.C.; Strasser, A.; Adams, J.M. Degenerative Disorders Caused by
557 Bcl-2 Deficiency Prevented by Loss of Its BH3-Only Antagonist Bim. *Dev. Cell.* **2001**, 1, 645–653.
- 558 5. Akhtar, R.S.; Ness, J.M.; Roth, K.A. Bcl-2 Family Regulation of Neuronal Development and
559 Neurodegeneration. *Biochim. Biophys. Acta* **2004**, 1644, 189–203.
- 560 6. Rodriguez-Martinez, J.A.; Reinke, A.W.; Bhimsaria, D.; Keating, A.E.; Ansari, A.Z. Combinatorial
561 bZIP Dimers Define Complex DNA-Binding Specificity Landscapes. *eLife* **2017**, 6, e19272.
- 562 7. Lessene, G.; Czabotar, P.E.; Colman, P.M. BCL-2 Family Antagonists for Cancer Therapy. *Nat.*
563 *Rev. Drug. Discov.* **2008**, 7, 989–1000.
- 564 8. Souers, A.J.; Levenson, J.D.; Boghaert, E.R.; Ackler, S.L.; Catron, N.D.; Chen, J.; Dayton, B.D.;
565 Ding, H.; Enschede, S.H.; Fairbrother, W.J.; Huang, D.C.; Hymowitz, S.G.; Jin, S.; Khaw, S.L.; Kovar, P.J.;
566 Lam, L.T.; Lee, J.; Maecker, H.L.; Marsh, K.C.; Mason, K.D.; Mitten, M.J.; Nimmer, P.M.; Oleksijew, A.;
567 Park, C.H.; Park, C.M.; Phillips, D.C.; Roberts, A.W.; Sampath, D.; Seymour, J.F.; Smith, M.L.; Sullivan,
568 G.M.; Tahir, S.K.; Tse, C.; Wendt, M.D.; Xiao, Y.; Xue, J.C.; Zhang, H.; Humerickhouse, R.A.; Rosenberg,
569 S.H.; Elmore, S.W. ABT-199, a Potent and Selective BCL-2 Inhibitor, Achieves Antitumor Activity While
570 Sparing Platelets. *Nat. Med.* **2013**, 19, 202–208.
- 571 9. Cohen, P.; Tcherpakov, M. Will the Ubiquitin System Furnish as Many Drug Targets as Protein
572 Kinases? *Cell* **2010**, 143, 686–693.

- 573 10. Gotoh, Y.; Eguchi, Y.; Watanabe, T.; Okamoto, S.; Doi, A.; Utsumi, R. Two-Component Signal
574 Transduction as Potential Drug Targets in Pathogenic Bacteria. *Curr. Opin. Microbiol.* **2010**, *13*, 232–239.
- 575 11. Siryk-Bathgate, A.; Dabul, S.; Lymperopoulos, A. Current and Future G Protein-Coupled Receptor
576 Signaling Targets for Heart Failure Therapy. *Drug. Des. Devel. Ther.* **2013**, *7*, 1209–1222.
- 577 12. Curtis, R.A.; Lue, L. A Molecular Approach to Bioseparations: Protein–Protein and Protein–Salt
578 Interactions. *Chem. Eng. Sci.* **2006**, *61*, 907–923.
- 579 13. Velev, O.D.; Kaler, E.W.; Lenhoff, A.M. Protein Interactions in Solution Characterized by Light and
580 Neutron Scattering: Comparison of Lysozyme and Chymotrypsinogen. *Biophys. J.* **1998**, *75*, 2682–2697.
- 581 14. Gromiha, M.M.; Yokota, K.; Fukui, K. Energy Based Approach for Understanding the Recognition
582 Mechanism in Protein-Protein Complexes. *Mol. Biosyst.* **2009**, *5*, 1779–1786.
- 583 15. Gromiha, M.M.; Saranya, N.; Selvaraj, S.; Jayaram, B.; Fukui, K. Sequence and Structural Features
584 of Binding Site Residues in Protein-Protein Complexes: Comparison with Protein-Nucleic Acid Complexes.
585 *Proteome Sci.* **2011**, *9* (Suppl 1), S13.
- 586 16. Aiello, D.; Caffrey, D.R. Evolution of Specific Protein-Protein Interaction Sites Following Gene
587 Duplication. *J. Mol. Biol.* **2012**, *423*, 257–272.
- 588 17. Ivanov, S.M.; Huber, R.G.; Warwicker, J.; Bond, P.J. Energetics and Dynamics Across the Bcl-2-
589 Regulated Apoptotic Pathway Reveal Distinct Evolutionary Determinants of Specificity and Affinity.
590 *Structure* **2016**, *24*, 2024–2033.
- 591 18. Cuthbertson, J.M.; Bond, P.J.; Sansom, M.S.P. Transmembrane Helix-Helix Interactions:
592 Comparative Simulations of the Glycophorin A Dimer. *Biochemistry* **2006**, *45*, 14298–14310.
- 593 19. Vicini, P.; Zani, F.; Cozzini, P.; Doytchinova, I. Hydrazones of 1,2-Benzisothiazole Hydrazides:
594 Synthesis, Antimicrobial Activity and QSAR Investigations. *Eur. J. Med. Chem.* **2002**, *37*, 553–564.
- 595 20. Sushko, I.; Novotarskyi, S.; Körner, R.; Pandey, A.K.; Kovalishyn, V.V.; Prokopenko, V.V.; Tetko,
596 I.V. Applicability Domain for in Silico Models to Achieve Accuracy of Experimental Measurements. *J.*
597 *Chemom.* **2010**, *24*, 202–208.
- 598 21. Ivanov, S.; Dimitrov, I.; Doytchinova, I. Quantitative Prediction of Peptide Binding to HLA-DP1

599 Protein. *IEEE/ACM Trans. Comput. Biol. Bioinform.* **2013**, *10*, 811–815.

600 22. Patronov, A.; Dimitrov, I.; Flower, D.R.; Doytchinova, I. Peptide Binding to HLA-DP Proteins at pH
601 5.0 and pH 7.0: a Quantitative Molecular Docking Study. *BMC Struct. Biol.* **2012**, *12*, 20-34.

602 23. Patronov, A.; Doytchinova, I. T-Cell Epitope Vaccine Design by Immunoinformatics. *Open Biol.*
603 **2013**, *3*, 120139.

604 24. Andrade, C.H.; Pasqualoto, F.M.; Ferreira, E.I.; Hopfinger, A.J. 4D-QSAR: Perspectives in Drug
605 Design. *Molecules* **2010**, *15*, 3281-3294.

606 25. Varnes, J.G.; Gero, T.; Huang, S.; Diebold, R.B.; Ogoe, C.; Grover, P.T.; Su, M.; Mukherjee, P.;
607 Saeh, J.C.; MacIntyre, T.; Repik, G.; Dillman, K.; Byth, K.; Russell, D.J.; Ioannidis, S. Towards the Next
608 Generation of Dual Bcl-2/Bcl-xL Inhibitors. *Bioorganic Med. Chem. Lett.* **2014**, *24*, 3026–3033.

609 26. Moldoveanu, T.; Follis, A.V.; Kriwacki, R.W.; Green, D.R. Many Players in BCL-2 Family Affairs.
610 *Trends Biochem. Sci.* **2014**, *39*, 101–111.

611 27. Ng, S.Y.; Davids, M.S. Selective Bcl-2 Inhibition to Treat Chronic Lymphocytic Leukemia and
612 Non-Hodgkin Lymphoma. *Clin. Adv. Hematol. Oncol.* **2014**, *12*, 224–229.

613 28. Gandhi, L.; Camidge, D.R.; De Oliveira, M.R.; Bonomi, P.; Gandara, D.; Khaira, D.; Hann, C.L.;
614 McKeegan, E.M.; Litvinovich, E.; Hemken, P.M.; Dive, C.; Enschede, S.H.; Nolan, C.; Chiu, YL.; Busman,
615 T.; Xiong, H.; Krivoshik, A.P.; Humerickhouse, R.; Shapiro, G.I.; Rudin, C.M. Phase I Study of Navitoclax
616 (ABT-263), a Novel Bcl-2 Family Inhibitor, in Patients with Small-Cell Lung Cancer and Other Solid
617 Tumors. *J. Clin. Oncol.* **2011**, *29*, 909–916.

618 29. Okamoto, T.; Campbell, S.; Mehta, N.; Thibault, J.; Colman, P.M.; Barry, M.; Huang, D.C.S.;
619 Kvensakul, M. Sheeppox Virus SPPV14 Encodes a Bcl-2-Like Cell Death Inhibitor That Counters a Distinct
620 Set of Mammalian Proapoptotic Proteins. *J. Virol.* **2012**, *86*, 11501–11511.

621 30. Wendt, M.D.; Shen, W.; Kunzer, A.; McClellan, W.J.; Bruncko, M.; Oost, T.K.; Ding, H.; Joseph,
622 M.K.; Zhang, H.; Nimmer, P.M.; Ng, S.C.; Shoemaker, A.R.; Petros, A.M.; Oleksijew, A.; Marsh, K.;
623 Bauch, J.; Oltersdorf, T.; Belli, B.A.; Martineau, D.; Fesik, S.W.; Rosenberg, S.H.; Elmore, S.W. Discovery
624 and Structure- Activity Relationship of Antagonists of B-Cell Lymphoma 2 Family Proteins with
625 Chemopotential Activity in Vitro and in Vivo. *J. Med. Chem.* **2006**, *49*, 1165–1181.

- 626 31. Bruncko, M.; Oost, T.K.; Belli, B.A.; Ding, H.; Joseph, M.K.; Kunzer, A.; Martineau, D.;
627 McClellan, W.J.; Mitten, M.; Ng, S.C.; Nimmer, P.M.; Oltersdorf, T.; Park, C.M.; Petros, A.M.; Shoemaker,
628 A.R.; Song, X.; Wang, X.; Wendt, M.D.; Zhang, H.; Fesik, S.W.; Rosenberg, S.H.; Elmore, S.W. Studies
629 Leading to Potent, Dual Inhibitors of Bcl-2 and Bcl-xL. *J. Med. Chem.* **2007**, *50*, 641–662.
- 630 32. Porter, J.; Payne, A.; de Candole, B.; Ford, D.; Hutchinson, B.; Trevitt, G.; Turner, J.; Edwards, C.;
631 Watkins, C.; Whitcombe, I.; Davis, J.; Stubberfield, C. Tetrahydroisoquinoline Amide Substituted Phenyl
632 Pyrazoles as Selective Bcl-2 Inhibitors. *Bioorganic Med. Chem. Lett.* **2009**, *19*, 230–233.
- 633 33. Tao, Z.; Hasvold, L.; Wang, L.; Wang, X.; Petros, A.M.; Park, C.H.; Boghaert, E.R.; Catron, N.D.;
634 Chen, J.; Colman, P.M.; Czabotar, P.E.; Deshayes, K.; Fairbrother, W.J.; Flygare, J.A.; Hymowitz, S.G.; Jin,
635 S.; Judge, R.A.; Koehler, M.F.; Kovar, P.J.; Lessene, G.; Mitten, M.J.; Ndubaku, C.O.; Nimmer, P.; Purkey,
636 H.E.; Oleksijew, A.; Phillips, D.C.; Sleebs, B.E.; Smith, B.J.; Smith, M.L.; Tahir, S.K.; Watson, K.G.; Xiao,
637 Y.; Xue, J.; Zhang, H.; Zobel, K.; Rosenberg, S.H.; Tse, C.; Levenson, J.D.; Elmore, S.W.; Souers, A.J.
638 Discovery of a Potent and Selective BCL - XL Inhibitor with in Vivo Activity. *ACS Med. Chem. Lett.* **2014**,
639 *5*, 1088–1093.
- 640 34. Rudin, C.M.; Hann, C.L.; Garon, E.B.; Ribeiro De Oliveira, M.; Bonomi, P.D.; Camidge, D.R.; Chu,
641 Q.; Giaccone, G.; Khaira, D.; Ramalingam, S.S.; Ranson, M.R.; Dive, C.; McKeegan, E.M.; Chyla, B.J.;
642 Dowell, B.L.; Chakravartty, A.; Nolan, C.E.; Rudersdorf, N.; Busman, T.A.; Mabry, M.H.; Krivoschik, A.P.;
643 Humerickhouse, R.A.; Shapiro, G.I.; Gandhi, L. Phase II Study of Single-Agent Navitoclax (ABT-263) and
644 Biomarker Correlates in Patients with Relapsed Small Cell Lung Cancer. *Clin. Cancer Res.* **2012**, *18*, 3163–
645 3169.
- 646 35. Sleebs, B.E.; Czabotar, P.E.; Fairbrother, W.J.; Fairlie, W.D.; Flygare, J.A.; Huang, D.C.S.; Kersten,
647 W.J.; Koehler, M.F.; Lessene, G.; Lowes, K.; Parisot, J.P.; Smith, B.J.; Smith, M.L.; Souers, A.J.; Street,
648 I.P.; Yang, H.; Baell, J.B. Quinazoline Sulfonamides as Dual Binders of the Proteins B-Cell Lymphoma 2
649 and B-Cell Lymphoma Extra Long with Potent Proapoptotic Cell-Based Activity. *J. Med. Chem.* **2011**, *54*,
650 1914–1926.
- 651 36. Rocklin, G.J.; Boyce, S.E.; Fischer, M.; Fish, I.; Mobley, D.L.; Shoichet, B.K.; Dill, K.A. Blind
652 Prediction of Charged Ligand Binding Affinities in a Model Binding Site. *J. Mol. Biol.* **2013**, *425*, 4569–
653 4583.

- 654 37. Lee, E.F.; Czabotar, P.E.; Yang, H.; Sleebs, B.E.; Lessene, G.; Colman, P.M.; Smith, B.J.; Fairlie,
655 W.D. Conformational Changes in Bcl-2 Pro-Survival Proteins Determine their Capacity to Bind Ligands. *J.*
656 *Biol. Chem.* **2009**, *284*, 30508–30517.
- 657 38. Perez, H.L.; Banfi, P.; Bertrand, J.; Cai, Z.W.; Grebinski, J.W.; Kim, K.; Lippy, J.; Modugno, M.;
658 Naglich, J.; Schmidt, R.J.; Tebben, A.; Vianello, P.; Wei, D.D.; Zhang, L.; Galvani, A.; Lombardo, L.J.;
659 Borzilleri, R.M. Identification of a Phenylacetylsulfonamide Series of Dual Bcl-2/Bcl-xL Antagonists.
660 *Bioorganic Med. Chem. Lett.* **2012**, *22*, 3946–3950.
- 661 39. Zhou, H.; Chen, J.; Meagher, J.L.; Yang, C.Y.; Aguilar, A.; Liu, L.; Bai, L.; Cong, X.; Cai, Q.; Fang,
662 X.; Stuckey, J.A.; Wang, S. Design of Bcl-2 and Bcl-xL Inhibitors with Subnanomolar Binding Affinities
663 Based upon a New Scaffold. *J. Med. Chem.* **2012**, *55*, 4664–4682.
- 664 40. Sleebs, B.E.; Kersten, W.J.A.; Kulasegaram, S.; Nikolakopoulos, G.; Hatzis, E.; Moss, R.M.;
665 Parisot, J.P.; Yang, H.; Czabotar, P.E.; Fairlie, W.D.; Lee, E.F.; Adams, J.M.; Chen, L.; van Delft, M.F.;
666 Lowes, K.N.; Wei, A.; Huang, D.C.; Colman, P.M.; Street, I.P.; Baell, J.B.; Watson, K.; Lessene, G.
667 Discovery of Potent and Selective Benzothiazole Hydrazone Inhibitors of Bcl-XL. *J. Med. Chem.* **2013**, *56*,
668 5514–5540.
- 669 41. Levenson, J.D.; Phillips, D.C.; Mitten, M.J.; Boghaert, E.R.; Diaz, D.; Tahir, S.K.; Belmont, L.D.;
670 Nimmer, P.; Xiao, Y.; Ma, X.M.; Lowes, K.N.; Kovar, P.; Chen, J.; Jin, S.; Smith, M.; Xue, J.; Zhang, H.;
671 Oleksijew, A.; Magoc, T.J.; Vaidya, K.S.; Albert, D.H.; Tarrant, J.M.; La, N.; Wang, L.; Tao, Z.F.; Wendt,
672 M.D.; Sampath, D.; Rosenberg, S.H.; Tse, C.; Huang, D.C.; Fairbrother, W.J.; Elmore, S.W.; Souers, A.J.
673 Exploiting Selective BCL-2 Family Inhibitors to Dissect Cell Survival Dependencies and Define Improved
674 Strategies for Cancer Therapy. *Sci. Transl. Med.* **2015**, *7*, 279ra40.
- 675 42. Yusuff, N.; Doré, M.; Joud, C.; Visser, M.; Springer, C.; Xie, X.; Herlihy, K.; Porter, D.; Touré,
676 B.B. Lipophilic Isosteres of a π - π Stacking Interaction: New Inhibitors of the Bcl-2-Bak Protein-Protein
677 Interaction. *ACS Med. Chem. Lett.* **2012**, *3*, 579–583.
- 678 43. Brady, R.M.; Vom, A.; Roy, M.J.; Toovey, N.; Smith, B.J.; Moss, R.M.; Hatzis, E.; Huang, D.C.;
679 Parisot, J.P.; Yang, H.; Street, I.P.; Colman, P.M.; Czabotar, P.E.; Baell, J.B.; Lessene, G. De-Novo
680 Designed Library of Benzoylureas as Inhibitors of BCL-XL: Synthesis, Structural and Biochemical

681 Characterization. *J. Med. Chem.* **2014**, *57*, 1323–1343.

682 44. Hennessy, E.J. Selective inhibitors of Bcl-2 and Bcl-xL: Balancing Antitumor Activity with On-
683 Target Toxicity. *Bioorganic Med. Chem Lett.* **2016**, *26*, 2105–2114.

684 45. Lessene, G.; Czabotar, P.E.; Sleebs, B.E.; Zobel, K.; Lowes, K.N.; Adams, J.M.; Baell, J.B.;
685 Colman, P.M.; Deshayes, K.; Fairbrother, W.J.; Flygare, J.A.; Gibbons, P.; Kersten, W.J.; Kulasegaram, S.;
686 Moss, R.M.; Parisot, J.P.; Smith, B.J.; Street, I.P.; Yang, H.; Huang, D.C.; Watson, K.G. Structure-Guided
687 Design of a Selective BCL-XL Inhibitor. *Nat. Chem. Biol.* **2013**, *9*, 390–397.

688 46. Feng, Y.; Ding, X.; Chen, T.; Chen, L.; Liu, F.; Jia, X.; Luo, X.; Shen, X.; Chen, K.; Jiang, H.;
689 Wang, H.; Liu, H.; Liu, D. Design, Synthesis, and Interaction Study of Quinazoline-2(1H)-Thione
690 Derivatives as Novel Potential Bcl-xL Inhibitors. *J. Med. Chem.* **2010**, *53*, 3465–3479.

691 47. Aguilar, A.; Zhou, H.; Chen, J.; Liu, L.; Bai, L.; Mceachern, D.; Yang, C.Y.; Meagher, J.; Stuckey,
692 J.; Wang, S. A Potent and Highly Efficacious Bcl-2/Bcl-xL Inhibitor. *J. Med. Chem.* **2013**, *56*, 3048–3067.

693 48. Park, C.M.; Bruncko, M.; Adickes, J.; Bauch, J.; Ding, H.; Kunzer, A.; Marsh, K.C.; Nimmer, P.;
694 Shoemaker, A.R.; Song, X.; Tahir, S.K.; Tse, C.; Wang, X.; Wendt, M.D.; Yang, X.; Zhang, H.; Fesik, S.W.;
695 Rosenberg, S.H.; Elmore, S.W. Discovery of an Orally Bioavailable Small Molecule Inhibitor of Prosurvival
696 B-Cell Lymphoma 2 Proteins. *J. Med. Chem.* **2008**, *51*, 6902–6915.

697 49. Touré, B.B.; Miller-Moslin, K.; Yusuff, N.; Perez, L.; Doré, M.; Joud, C.; Michael, W.; DiPietro, L.;
698 van der Plas, S.; McEwan, M.; Lenoir, F.; Hoe, M.; Karki, R.; Springer, C.; Sullivan, J.; Levine, K.; Fiorilla,
699 C.; Xie, X.; Kulathila, R.; Herlihy, K.; Porter, D.; Visser, M. The Role of the Acidity of N-Heteroaryl
700 Sulfonamides as Inhibitors of Bcl-2 Family Protein-Protein Interactions. *ACS Med. Chem. Lett.* **2013**, *4*,
701 186–190.

702 50. Vogler, M.; Dinsdale, D.; Dyer, M.J.S.; Cohen, G.M. Bcl-2 Inhibitors: Small Molecules with a Big
703 Impact on Cancer Therapy. *Cell Death Differ.* **2009**, *16*, 360–367.

704 51. Zhai, D.; Jin, C.; Satterthwait, A.C.; Reed, J.C. Comparison of Chemical Inhibitors of Antiapoptotic
705 Bcl-2-Family Proteins. *Cell Death Differ.* **2006**, *13*, 1419–1421.

706 52. Dror, R.O.; Dirks, R.M.; Grossman, J.P.; Xu, H.; Shaw, D.E. Biomolecular Simulation: a
707 Computational Microscope for Molecular Biology. *Annu. Rev. Biophys.* **2012**, *41*, 429–452.

- 708 53. Gasteiger, J.; Marsili, M. Iterative Partial Equalization of Orbital Electronegativity - a Rapid Access
709 to Atomic Charges. *Tetrahedron* **1980**, *36*, 3219–3228.
- 710 54. Balaban, A.T. Highly Discriminating Distance-Based Topological index. *Chem. Phys. Lett.* **1982**,
711 *89*, 399–404.
- 712 55. Bertz, S. The First General Index of Molecular Complexity. *J. Am. Chem. Soc.* **1981**, *83*, 3599–3601.
- 713 56. Bonchev, D.; Trinajstić, N. Information Theory, Distance Matrix, and Molecular Branching. *J.*
714 *Chem. Phys.* **1977**, *67*, 4517–4533.
- 715 57. Hall, L.H.; Kier, L.B. The Molecular Connectivity Chi Indexes and Kappa Shape Indexes in
716 Structure-Property Modeling. *Rev. Comput. Chem.* **1991**, *2*, 367–422.
- 717 58. Wildman, S.; Crippen, G. Prediction of Physicochemical Parameters by Atomic Contributions. *J.*
718 *Chem. Inf. Model.* **1999**, *39*, 868–873.
- 719 59. Ertl, P.; Rohde, B.; Selzer, P. Fast Calculation of Molecular Polar Surface Area as a Sum of
720 Fragment-Based Contributions and its Application to the Prediction of Drug Transport Properties. *J. Med.*
721 *Chem.* **2000**, *43*, 3714–3717.
- 722 60. Labute, P. A Widely Applicable Set of Descriptors. *J. Mol. Graph. Model.* **2000**, *18*, 464–477.
- 723 61. Nguyen, K.T.; Blum, L.C.; van Deursen, R.; Reymond, J-L. Classification of Organic Molecules by
724 Molecular Quantum Numbers. *ChemMedChem.* **2009**, *4*, 1803–1805.
- 725 62. Gramatica, P. Principles of QSAR Models Validation: Internal and External. *QSAR Comb. Sci.* **2007**,
726 *26*, 694–701.
- 727 63. Mitchell, J.B.O. Machine Learning Methods in Chemoinformatics. *Wiley Interdiscip. Rev.: Comput.*
728 *Mol. Sci.* **2014**, *4*, 468–481.
- 729 64. Gu, M.; Lai, T.L. Jackknife. Bootstrap and Other Resampling Methods in Regression Analysis. *Ann.*
730 *Stat.* **1991**, *19*, 1403–1433.
- 731 65. Mevik, B-H.; Wehrens, R. The pls Package: Principle Component and Partial Least Squares
732 Regression in R. *J. Stat. Softw.* **2007**, *18*, 1–24.
- 733 66. Brandsdal, B.O.; Österberg, F.; Almlöf, M.; Freierberg, I.; Luzhkov, V.B.; Åqvist, J. Free Energy

734 Calculations and Ligand Binding. *Adv. Protein Chem.* **2003**, *66*, 123–158.

735 67. Hansen, N.; van Gunsteren, W.F. Practical Aspects of Free-Energy Calculations: a Review. *J. Chem.*
736 *Theory Comput.* **2014**, *10*, 2632–2647.

737 68. Case, D.A.; Cheatham, T.E. 3rd; Darden, T.; Gohlke, H.; Luo, R.; Merz, K.M. Jr.; Onufriev, A.;
738 Simmerling, C.; Wang, B.; Woods, R.J. The Amber Biomolecular Simulation Programs. *J. Comput. Chem.*
739 **2005**, *26*, 1668–1688.

740 69. Jorgensen, W.L.; Chandrasekhar, J.; Madura, J.D.; Impey, R.W.; Klein, M.L. Comparison of Simple
741 Potential Functions for Simulating Liquid Water. *J. Chem. Phys.* **1983**, *79*, 926.

742 70. Dixon, S.L.; Jurs, P.C. Estimation of pKa for Organic Oxyacids using Calculated Atomic Charges. *J.*
743 *Comput. Chem.* **1993**, *14*, 1460–1467.

744 71. Csizmadia, F.; Tsantili-Kakoulidou, A.; Panderi, I.; Darvas, F. Prediction of Distribution Coefficient
745 from Structure. 1. Estimation Method. *J. Pharm. Sci.* **1997**, *86*, 865–871.

746 72. Wang, J.M.; Wolf, R.M.; Caldwell, J.W.; Kollman, P.A.; Case, D.A. Development and Testing of a
747 General Amber Force Field. *J. Comput. Chem.* **2004**, *25*, 1157–1174.

748 73. Jakalian, A.; Bush, B.L.; Jack, D.B.; Bayly, C.I. Fast, Efficient Generation of High-Quality Atomic
749 Charges. AM1-BCC Model: I. Method. *J. Comput. Chem.* **2000**, *21*, 132–146.

750 74. Berendsen, H.J.C.; Postma, J.P.M.; van Gunsteren, W.F.; DiNola, A.; Haak, J.R. Molecular
751 Dynamics with Coupling to an External Bath. *J. Chem. Phys.* **1984**, *81*, 3684–3690.

752 75. Adelman, S.A.; Doll, J.D. Generalized Langevin Equation Approach for Atom/Solid–Surface
753 Scattering: Collinear Atom/Harmonic Chain Model. *J. Chem. Phys.* **1974**, *61*, 4242–4245.

754 76. Darden, T.; York, D.; Pedersen, L. Particle Mesh Ewald: An $N \log(N)$ Method for Ewald Sums in
755 Large Systems. *J. Chem. Phys.* **1993**, *98*, 10089.

756 77. Ciccotti, G.; Ryckaert, J.P. Molecular Dynamics Simulation of Rigid Molecules. *Comput. Phys.*
757 *Reports* **1986**, *4*, 346–392.

758 78. Roe, D.R.; Cheatham, T.E. 3rd. PTRAJ and CPPTRAJ: Software for Processing and Analysis of
759 Molecular Dynamics Trajectory Data. *J. Chem. Theory Comput.* **2013**, *9*, 3084–3095.

- 760 79. Wang, L.; Wu, Y.; Deng, Y.; Kim, B.; Pierce, L.; Krilov, G.; Lupyan, D.; Robinson, S.; Dahlgren,
761 M.K.; Greenwood, J.; Romero, D.L.; Masse, C.; Knight, J.L.; Steinbrecher, T.; Beuming, T.; Damm, W.;
762 Harder, E.; Sherman, W.; Brewer, M.; Wester, R.; Murcko, M.; Frye, L.; Farid, R.; Lin, T.; Mobley, D.L.;
763 Jorgensen, W.L.; Berne, B.J.; Friesner, R.A.; Abel, R. Accurate and Reliable Prediction of Relative Ligand
764 Binding Potency in Prospective Drug Discovery by way of a Modern Free-Energy Calculation Protocol and
765 Force Field. *J. Am. Chem. Soc.* **2015**, 137, 2695–2703.
- 766 80. Schrödinger Release 2017-2: Maestro, Schrödinger, LLC, New York, NY, **2017**.
- 767 81. Hornak, V.; Simmerling, C. Development of Softcore Potential Functions for Overcoming Steric
768 Barriers in Molecular Dynamics Simulations. *J. Mol. Graph. Model.* **2004**, 22, 405–413.
- 769 82. Steinbrecher, T.; Joung, I.; Case, D.A. Soft-Core Potentials in Thermodynamic Integration:
770 Comparing One- and Two-Step Transformations. *J. Comput. Chem.* **2011**, 32, 3253–3263.
- 771 83. Mobley, D.L.; Graves, A.P.; Chodera, J.D.; McReynolds, A.C.; Shoichet, B.K.; Dill, K.A. Predicting
772 Absolute Ligand Binding Free Energies to a Simple Model Site. *J. Mol. Biol.* **2007**, 371, 1118–1134.
- 773 84. Klimovich, P.V.; Shirts, M.R.; Mobley, D.L. Guidelines for the Analysis of Free Energy
774 Calculations. *J. Comput. Aided Mol. Des.* **2015**, 29, 397–411.
- 775 85. Kollman, P. Free-Energy Calculations - Applications to Chemical and Biochemical Phenomena.
776 *Chem. Rev.* **1993**, 93, 2395–2417.
- 777 86. Steinbrecher, T.; Case, D.A.; Labahn, A. A Multistep Approach to Structure-Based Drug Design:
778 Studying Ligand Binding at the Human Neutrophil Elastase. *J. Med. Chem.* **2006**, 49, 1837–1844.
- 779 87. Gilson, M.K.; Zhou, H-X. Calculation of Protein-Ligand Binding Affinities. *Annu. Rev. Biophys.*
780 *Biomol. Struct.* **2007**, 36, 21–42.
- 781 88. Steinbrecher, T.; Hrenn, A.; Dormann, K.L.; Merfort, I.; Labahn, A. Bornyl (3,4,5-Trihydroxy)-
782 Cinnamate - an Optimized Human Neutrophil Elastase Inhibitor Designed by Free Energy Calculations.
783 *Bioorganic Med. Chem.* **2008**, 16, 2385–2390.
- 784 89. Kaus, J.W.; Pierce, L.T.; Walker, R.C.; McCammon, J.A. Improving the Efficiency of Free Energy
785 Calculations in the Amber Molecular Dynamics Package. *J. Chem. Theory Comput.* **2013**, 9, 1–8.

- 786 90. Murdoch, D.J.; Chow, E.D. A Graphical Display of Large Correlation Matrices. *Am. Stat.* **1996**, *50*,
787 178–180.
- 788 91. Friendly, M. Corrgrams: Exploratory Displays for Correlation Matrices. *Am. Stat.* **2002**, *34*, 1447–
789 1449.
- 790 92. Durant, J.L.; Leland, B.A.; Henry, D.R.; Nourse, J.G. Reoptimization of MDL Keys for Use in Drug
791 Discovery. *J. Chem. Inf. Model.* **2002**, *42*, 1273–1280.
- 792 93. Chodera, J.D.; Mobley, D.L.; Shirts, M.R.; Dixon, R.W.; Branson, K.; Pande, V.S. Alchemical Free
793 Energy Methods for Drug Discovery: Progress and Challenges. *Curr. Opin. Struct. Biol.* **2011**, *21*, 150–160.
- 794 94. Lee, E.F.; Czabotar, P.E.; Smith, B.J.; Deshayes, K.; Zobel, K.; Colman, P.M.; Fairlie, W.D. Crystal
795 Structure of ABT-737 Complexed with Bcl-xL: Implications for Selectivity of Antagonists of the Bcl-2
796 family. *Cell Death Differ.* **2007**, *14*, 1711–1713.
- 797 95. Oltersdorf, T.; Elmore, S.W.; Shoemaker, A.R.; Armstrong, R.C.; Augeri, D.J.; Belli, B.A.;
798 Bruncko, M.; Deckwerth, T.L.; Dinges, J.; Hajduk, P.J.; Joseph, M.K.; Kitada, S.; Korsmeyer, S.J.; Kunzer,
799 A.R.; Letai, A.; Li, C.; Mitten, M.J.; Nettesheim, D.G.; Ng, S.; Nimmer, P.M.; O'Connor, J.M.; Oleksijew,
800 A.; Petros, A.M.; Reed, J.C.; Shen, W.; Tahir, S.K.; Thompson, C.B.; Tomaselli, K.J.; Wang, B.; Wendt,
801 M.D.; Zhang, H.; Fesik, S.W.; Rosenberg, S.H. An Inhibitor of Bcl-2 Family Proteins Induces Regression of
802 Solid Tumours. *Nature* **2005**, *435*, 677–681.
- 803 96. Tanaka, Y.; Aikawa, K.; Nishida, G. Discovery of Potent Mcl-1/Bcl-xL Dual Inhibitors by Using a
804 Hybridization Strategy Based on Structural Analysis of Target Proteins. *J. Med. Chem.* **2013**, *56*, 9635–9645.
- 805 97. Mobley, D.L.; Klimovich, P.V. Perspective: Alchemical Free Energy Calculations for Drug
806 Discovery. *J. Chem. Phys.* **2012**, *137*, 230901.
- 807 98. Burusco, K.K.; Bruce, N.J.; Alibay, I.; Bryce, R.A. Free Energy Calculations using a Swarm-
808 Enhanced Sampling Molecular Dynamics Approach. *ChemPhysChem.* **2015**, *16*, 3233–3241.
- 809 99. Lyne, P.D.; Lamb, M.L.; Saeh, J.C. Accurate Prediction of the Relative Potencies of Members of a
810 Series of Kinase Inhibitors Using Molecular Docking and MM-GBSA Scoring. *J. Med. Chem.* **2006**, *49*,
811 4805–4808.

- 812 100. Settimo, L.; Bellman, K.; Knegtel, R.M.A. Comparison of the Accuracy of Experimental and
813 Predicted pKa Values of Basic and Acidic Compounds. *Pharm. Res.* **2014**, *31*, 1082–1095.
- 814 101. Homeyer, N.; Stoll, F.; Hillisch, A.; Gohlke, H. Binding Free Energy Calculations for Lead
815 Optimization: Assessment of Their Accuracy in an Industrial Drug Design Context. *J. Chem. Theory*
816 *Comput.* **2014**, *10*, 1–31.
- 817 102. Aldeghi, M.; Heifetz, A.; Bodkin, M.J.; Knapp, S.; Biggin, P.C. Predictions of Ligand Selectivity
818 from Absolute Binding Free Energy Calculations. *J. Am. Chem. Soc.* **2016**, *139*, 946–957.
- 819 103. Christ, C.D.; Fox, T. Accuracy Assessment and Automation of Free Energy Calculations for Drug
820 Design. *J. Chem. Inf. Model.* **2014**, *54*, 108–120.
- 821 104. Rocklin, G.J.; Mobley, D.L.; Dill, K.A.; Hünenberger, P.H. Calculating the Binding Free Energies of
822 Charged Species Based on Explicit-Solvent Simulations Employing Lattice-Sum Methods: an Accurate
823 Correction Scheme for Electrostatic Finite-Size Effects. *J. Chem. Phys.* **2013**, *139*, 184103.
- 824 105. Liu, S.; Wu, Y.; Lin, T.; Abel, R.; Redmann, J.P.; Summa, C.M.; Jaber, V.R.; Lim, N.M.; Mobley,
825 D.L. Lead Optimization Mapper: Automating Free Energy Calculations for Lead Optimization. *J. Comput.*
826 *Aided Mol. Des.* **2013**, *27*, 755–870.
- 827 106. Boyce, S.E.; Mobley, D.L.; Rocklin, G.J.; Graves, A.P.; Dill, K.A.; Shoichet, B.K. Predicting Ligand
828 Binding Affinity with Alchemical Free Energy Methods in a Polar Model Binding Site. *J. Mol. Biol.* **2009**,
829 *394*, 747–763.
- 830 107. Biniashvili, T.; Schreiber, E.; Kliger, Y. Improving Classical Substructure-Based Virtual Screening
831 to Handle Extrapolation Challenges. *J. Chem. Inf. Model.* **2012**, *52*, 678–685.
- 832 108. Roy, K.; Kar, S.; Ambure, P. On a Simple Approach for Determining Applicability Domain of
833 QSAR Models. *Chemom. Intell. Lab. Syst.* **2015**, *145*, 22–29.
- 834 109. Fourches, D.; Muratov, E.; Ding, F.; Dokholyan, N.V.; Tropsha, A. Predicting Binding Affinity of
835 CSAR Ligands Using Both Structure-Based and Ligand-Based Approaches. *J. Chem. Inf. Model.* **2013**, *53*,
836 1915–1922.
- 837 110. Cruz-Monteagudo, M.; Medina-Franco, J.L.; Perez-Castillo, Y.; Nicolotti, O.; Cordeiro, M.N.D.S.;

838 Borges, F. Activity Cliffs in Drug Discovery: Dr Jekyll or Mr Hyde? *Drug Discov. Today* **2014**, *19*, 1069–
839 1080.

840 111. Oprea, T.I. On the Information Content of 2D and 3D Descriptors for QSAR. *J. Braz. Chem. Soc.*
841 **2002**, *13*, 811–815.

842 112. Stefaniak, F. Prediction of Compounds Activity in Nuclear Receptor Signaling and Stress Pathway
843 Assays Using Machine Learning Algorithms and Low-Dimensional Molecular Descriptors. *Front. Environ.*
844 *Sci.* **2015**, *3*, 1–7.

845 113. Archontis, G.; Simonson, T.; Moras, D.; Karplus, M. Specific Amino Acid Recognition by Aspartyl-
846 tRNA Synthetase Studied by Free Energy Simulations. *J. Mol. Biol.* **1998**, *275*, 823–846.

847 114. Ash, J.; Fourches, D. Characterizing the Chemical Space of ERK2 Kinase Characterizing the
848 Chemical Space of ERK2 Kinase Inhibitors Using Descriptors Computed from Molecular Dynamics
849 Trajectories. *J. Chem. Inf. Model.* **2017**, *57*, 1286–1299.

850 115. Khedkar, V.M.; Joseph, J.; Pissurlenkar, R.; Saran, A.; Coutinho, E.C. How Good are Ensembles in
851 Improving QSAR Models? The case with eCoRIA. *J. Biomol. Struct. Dyn.* **2015**, *33*, 749–769.

852 116. Ahmed, M.H.; Spyraakis, F.; Cozzini, P.; Tripathi, P.K.; Mozzarelli, A.; Scarsdale, J.N.; Safo, M.A.;
853 Kellogg, G.E. Bound Water at Protein-Protein Interfaces: Partners, Roles and Hydrophobic Bubbles as a
854 Conserved Motif. *PLoS One* **2011**, *6*, e24712.

855 117. Stegmann, C.M.; Seeliger, D.; Sheldrick, G.M.; De Groot, B.L.; Wahl, M.C. The Thermodynamic
856 Influence of Trapped Water Molecules on a Protein-Ligand Interaction. *Angew. Chemie – Int. Ed.* **2009**, *48*,
857 5207–5210.

858 118. Nguyen, C.N.; Young, T.K.; Gilson, M.K. Grid Inhomogeneous Solvation Theory: Hydration
859 Structure and Thermodynamics of the Miniature Receptor Cucurbit[7]Uril. *J. Chem. Phys.* **2012**, *137*, 1–17.

860 119. Imai, T.; Kovalenko, A.; Hirata, F. Solvation Thermodynamics of Protein Studied by the 3D-RISM
861 Theory. *Chem. Phys. Lett.* **2004**, *395*, 1–6.

862 120. Pan, D.; Tseng, Y.; Hopfinger, A.J. Quantitative Structure-Based Design: Formalism and
863 Application of Receptor-Dependent RD-4D-QSAR Analysis to a Set of Glucose Analogue Inhibitors of

864 Glycogen Phosphorylase. *J. Chem. Inf. Comput. Sci.* **2003**, *43*, 1591–1607.

865 121. da Rocha Pita, S.S.; Albuquerque, M.G.; Rodrigues, C.R.; Castro, H.C.; Hopfinger, A.J. Receptor-
866 Dependent 4D-QSAR Analysis of Peptidemimetic Inhibitors of Trypanosoma cruzi Trypanothione
867 Reductase with Receptor-Based Alignment. *Chem. Biol. Drug Des.* **2012**, *79*, 740–748.

868 122. Ivanov, S.M.; Cawley, A.; Huber, R.G.; Bond, P.J.; Warwicker, J. Protein – Protein Interactions in
869 Paralogues: Electrostatics Modulates Specificity on a Conserved Steric Scaffold. *PLoS One* **2017**, *12*,
870 e0185928.

871 123. Haerder, E.; Damm, W.; Maple, J.; Wu, C.; Reboul, M.; Xiang, J.Y.; Wang, L.; Lupyan, D.;
872 Dahlgren, M.K.; Knight, J.L.; Kaus, J.W.; Cerutti, D.S.; Krilov, G.; Jorgensen, W.L.; Abel, R.; Friesner,
873 R.A. OPLS3: A Force Field Providing Broad Coverage of Drug-like Small Molecules and Proteins. *J. Chem.*
874 *Theory Comput.* **2016**, *2*, 281–296.

875 124. Loeffler, H.H.; Michel, J.; Woods, C. FESetup: Automating Setup for Alchemical Free Energy
876 Simulations. *J. Chem. Inf. Model.* **2015**, *55*, 2485–2490.

877 **Figure 1. Crystal structures of Bcl-xL and Bcl-2 bound to small molecule ligands.** Bcl-xL bound to
878 ABT-737 (left, PDB ID 2YXJ) and Bcl-2 bound to Navitoclax (ABT-263) (right, PDB ID 4LVT). The
879 proteins are shown in surface representation, the ligands are in stick representation with carbon atoms in
880 white, oxygen in red, nitrogen in blue, sulfur in yellow, chlorine in green, and fluorine in gray. Deep
881 hydrophobic pockets on the surface of the proteins, occupied by ligand moieties, are labeled with p2 and p4.

883 **Figure 2. Compounds included in QSAR analysis.** All compounds discussed in this work plotted by
884 experimentally measured specificity, molecular weight and computed logP (clogP). For clarity, Bcl-xL-
885 specific ligands, dual binders, and Bcl-2-specific compounds are colored differently and the specificity
886 region between -1 and 1 is highlighted. Compounds A, B, C, D, E, F,G, N3C (ABT-737), 1XJ (Navitoclax,
887 ABT-263), and 3, 4, 5, 6, 7, and 8 (Venetoclax, ABT-199) are labeled, shown in bold, and discussed further
888 in the text. N3C's specificity was computed from IC₅₀ values reported by Sleebs et al.³⁵ As the affinities of
889 compounds 5 and 8 for Bcl-xL and Bcl-2 were beyond the measuring sensitivity of the experimental setup
890 ($K_i < 1$ nM,³¹ $K_i < 0.01$ nM,⁸ respectively), provisional specificities for those compounds were computed
891 using values of 1 and 0.01 nM, respectively (also see Figure 3).

893 **Figure 3. Structures of template and modeled compounds.** Chemical formulae of the six model
894 compounds (labeled 3, 4, 5, 6, 7, and 8) and the N3C (ABT-737) and 1XJ (Navitoclax, ABT-263) template
895 compounds used in molecular dynamics simulations. Shown above every compound is its K_i value (in nM)
896 for Bcl-xL, followed by the K_i value for Bcl-2 (in nM). Compounds 3, 4, N3C, and 1XJ are potent dual
897 binders, compounds 5 and 6 are Bcl-xL selective, compounds 7 and 8 are Bcl-2 selective. The exact affinities
898 of compounds 5 for Bcl-xL and N3C and 8 for Bcl-2 are above the measurement limit of the experimental
899 procedures. Affinity data on compounds 3, 4, 5, and 6 is from Bruncko et al.,³¹ 7, 8 (Venetoclax, ABT-199),
900 1XJ, and N3C is from Souers et al.;⁸ IC₅₀ values for N3C (3/6.1 nM) are also reported in Sleebs et al.³⁵ Chiral
901 carbon atoms marked with a black asterisk (*) were modeled in the R-configuration; such were the
902 compounds used in the experimental affinity measurements. The chiral atom in compound 4, marked with a
903 red asterisk (*), was modeled in the S-configuration; its configuration is not specified in the relevant
904 publication. Thus, the S-configuration was chosen, as it shields the -CH₃ group from solvent to a greater

degree than the R-configuration. The nitrogen atoms in compounds 5 and 6, marked with a blue asterisk (*), were modeled in both the protonated and unprotonated state. Henceforth, results reported for compounds 5 and 6 correspond to the state where these nitrogens are unprotonated (ligand net charge +1), except where explicitly stated otherwise. The canonical groove of the antiapoptotic proteins lies below the piperazinyl and sulfonamidophenyl rings. Compounds 3, 4, 5, 6, 7, and 8 had their terminal phenyl groups oriented in pocket 2 of the antiapoptotic proteins (see Figure 1), and N and O atoms pointing outward into the solvent, with the exception of the spiro nitrogen of compound 4, which was also buried in the groove. The configurations of the spiro moieties of compounds 3 and 4 were modeled to be identical to the ones reported in the experimental study.³¹

Figure 4. 2D QSAR results. (A) R^2 between predicted and experimental specificity of the compounds in the external validation test sets plotted against the iteration number for the 20,000 iterations. N3C's specificity was computed from IC_{50} values reported in Sleebs et al.,³⁵ specificities for compounds 5 and 8 were computed using K_i values of 1^{31} and 0.01^8 nM for Bcl-xL and Bcl-2, respectively (see Figure 3). (B) Predicted specificity plotted against experimental values for the 47 compound test set. Values for compounds without marked standard deviations are from the first QSAR model, values for compounds 3, 4, 5, 6, 7, and 8, N3C, and 1XJ are averages over the 20,000 iterations; these do not differ significantly from values from the first model; also shown is the standard deviation of predicted specificities for these compounds over the 20,000 iterations. The $x = y$ line is shown in purple, also shown is the line of best fit from the initial model; the corresponding line equation and R^2 are in the top left corner. The region between -1 and 1 predicted vs experimental specificity is highlighted with a square box. (C) The same plot as in (B) prepared for the 15 compound test set.

Figure 5. Results from template equilibration simulations. Ca RMSDs (top), ligand heavy atom RMSDs (middle), and computed MM-PBSA enthalpies of interaction (bottom) for the four 100-ns replicas of the Bcl-xL – ABT-737 (left) and Bcl-2 – ABT-263 (right) template structures. The vertical black lines indicate the time points chosen to be templates for the free energy calculations. Snapshots at $t = 30$ ns and $t =$

21 ns from replicas 2 and 1 were chosen for the Bcl-xL – ABT-737 and Bcl-2 – ABT-263 complexes, respectively.

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Figure 6. Calculated free energies. (A) Computed ΔG s from the 11 λ -window alchemical transformations, averaged over the three replicas; standard deviations are also shown. Ligand transformations in complex with Bcl-xL or Bcl-2 are labeled “CMP,” transformations where the ligand is free in solution are labeled “LIG.” ΔG s were evaluated from 0 – 1500 ps and from 1500 to 2500 ps of production dynamics. For ease of comparison, *pmemd* results (labeled “Forward”) are multiplied by -1. Compounds 3, 4, 5, 6, 7, and 8 are prefixed with “N3” and “1X” to designate transformations from/to the N3C and 1XJ templates, respectively, i.e. N33 and 1X3 stand for transformations involving compound 3 using the N3C and 1XJ templates, analogously for N34 and 1X4, etc. (B) Computed $\Delta\Delta G$ s from the 11 λ -window alchemical transformations, averaged over the three replicas; standard deviations are also shown. Differences between ligand transformations in complex with Bcl-xL or Bcl-2 (labeled “CMP” in panel A) and transformations where the ligand is free in solution (labeled “LIG” in panel A) are labeled as “CMP - LIG,” also shown is the experimentally measured free energy difference (last bar for every compound). $\Delta\Delta G$ s were evaluated from 0 – 1500 ps and from 1500 to 2500 ps of production dynamics; computed values are directly comparable to experimental data. For ease of comparison, *pmemd* results (labeled “Forward”) are multiplied by -1. Compounds 3, 4, 5, 6, 7, and 8 are prefixed with “N3” and “1X” to designate transformations from/to the N3C and 1XJ templates, respectively, i.e. N33 and 1X3 stand for transformations involving compound 3 using the N3C and 1XJ templates, analogously for N34 and 1X4, etc.

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Figure 7. Pearson correlations between per-residue energy values. (A) Pearson correlation coefficients between the per-residue energy values of Bcl-xL for the three replicas of the *sander* transformations (top row), and ligand Tanimoto, topological fingerprint, and MACCS key similarities (bottom row). Compounds 3, 4, 5, 6, 7, and 8 are prefixed with “N3” to designate transformations to the N3C template for the alchemical transformations, i.e. N33 stands for transformations involving compound 3 using the N3C template, analogously for N34, N35, etc.; correlations have been calculated from all protein residues using energy values from the latter 1 ns of production dynamics in every λ -window. (B) Pearson correlation

961 coefficients between the per-residue energy values of Bcl-2 for the three replicas of the *sander*
962 transformations (top row), and ligand Tanimoto, topological fingerprint, and MACCS key similarities
963 (bottom row). Compounds 3, 4, 5, 6, 7, and 8 are prefixed with “1X” to designate transformations to the 1XJ
964 template for the alchemical transformations, i.e. 1X3 stands for transformations involving compound 3 using
965 the 1XJ template, analogously for 1X4, 1X5, etc.; correlations have been calculated from all protein residues
966 using energy values from the latter 1 ns of production dynamics in every λ -window.

967

968 **Figure 8. Pearson correlations between per-residue energy values.** (A) Pearson correlation coefficients
969 between the per-residue energy values of Bcl-xL and Bcl-2 for the three replicas of the *sander*
970 transformations. Compounds 3, 4, 5, 6, 7, and 8 are prefixed with “N3” or “1X” to designate transformations
971 to the N3C and 1XJ templates, respectively, i.e. N33 and 1X3 stand for transformations involving compound
972 3 using the N3C and 1XJ templates, analogously for N34 and 1X4, etc.; correlations have been calculated
973 from Bcl-xL residues 25 – 137 and Bcl-2 residues 27 – 139 using energy values from the latter 1 ns of
974 production dynamics in every λ -window. The energetic responses of Bcl-xL and Bcl-2 when bound to the
975 same ligand are compared (highlighted diagonals), as are the energetic responses between Bcl-xL and Bcl-2
976 bound to compounds 3 and 4, 5 and 6, 7 and 8 (squares lying on the diagonals), and compounds 5 and 6,
977 and 7 and 8 (off-diagonal squares). (B) Pearson correlation coefficients between the per-residue energy
978 values of Bcl-xL and Bcl-2 for the three replicas of the *sander* transformations. Compounds 3, 4, 5, 6, 7, and
979 8 are prefixed with “N3” or “1X” to designate transformations to the N3C and 1XJ templates, respectively,
980 i.e. N33 and 1X3 stand for transformations involving compound 3 using the N3C and 1XJ templates,
981 analogously for N34 and 1X4, etc.; correlations have been calculated from Bcl-xL residues 25 – 137 and
982 Bcl-2 residues 27 – 139 using energy values from the latter 1 ns of production dynamics in every λ -window.
983 Apart from the +1 protonation state, reported heretofore, compounds 5 and 6 are also examined in the +2
984 protonation state, where the piperazynyl nitrogen (see Figure 3) is protonated. The +1 and +2 states are
985 designated with a corresponding suffix in this panel.

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987 **Figure 9. 4D QSAR results.** (A) R^2 between predicted and experimental specificity of the compounds in
988 the external validation test sets plotted against the iteration number for the 20,000 iterations. Experimental

specificities for compounds 5 and 8 were computed using K_i values of 1^{31} and 0.01^8 nM for Bcl-xL and Bcl-2, respectively (see Figure 3). **(B)** Predicted specificity plotted against experimental values; training set consists of compounds 3, 5, and 7; test set includes ligands 4, 6, and 8; per-residue energy values for compounds 5 and 6 are from the +1 protonation state. Note that the training and test sets contain separate energy entries for the compounds bound to Bcl-xL and Bcl-2. Consequently, outputted predictions have two data points per compound; these are sufficiently close for meaningful analysis. This pertains to panels A and C as well. The values shown for the predicted specificities are averages over the 20,000 iterations; also shown are the standard deviations. The $x = y$ line is shown in purple, also shown is the line of best fit; the corresponding line equation and R^2 are in the top left corner. The region between -1 and 1 predicted vs experimental specificity is highlighted with a square box. **(C)** The same plot as in **(B)** prepared using ligands 4, 6, and 8 as the training set, and ligands 3, 5, and 7 as the test set; per-residue energy values for compounds 5 and 6 are from the +1 protonation state.