Protein–Ligand Binding

DOI: 10.1002/ange.201301813

The Binding of Benzoarylsulfonamide Ligands to Human Carbonic Anhydrase is Insensitive to Formal Fluorination of the Ligand**

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The hydrophobic effect (or the aggregated effects that we call "the hydrophobic effect") that underlies the binding of many ligands to proteins involves three molecular participants: the surface of the binding pocket of the protein, the surface of the ligand, and the networks of waters that fill the pocket and surround the ligand. The molecular-level mechanism of the hydrophobic effect in protein–ligand binding remains a subject of substantial controversy.[1–3] There are three primary questions of interest: 1) Do hydrophobic effects reflect the release of structured (entropically unfavorable) waters from hydrophobic surfaces when the ligand and surface of the binding pocket come into contact? 2) Do hydrophobic effects represent the displacement of free-energetically unfavorable waters from the binding pocket by the ligand, and the release of free-energetically unfavorable (although perhaps different) waters from the hydrophobic surface of the ligand? 3) How important in free energy are the contact interactions between the protein and the ligand?

In a previous examination of these questions,[4] we compared the binding of a series of heteroarylsulfonamide ligands, and their "benzo-extended" analogues (Scheme 1), to human carbonic anhydrase II (HCA; EC 4.2.1.1). The addition of a benzo group: 1) increased the hydrophobic surface area (and the volume) of the ligand; 2) generated new van der Waals contacts between the ligand and hydrophobic wall of HCA; but 3) did not result in a significant increase in the area of contact between the hydrophobic surfaces of the protein and ligand. The free energy of binding of the arylsulfonamide ligands increased by $-20\ \mathrm{cal\,mol^{-1}\,Å^{-2}}$ with the additional surface area of the benzo-extension,[4] an amount expected for normal hydrophobic effects (-20 to $-33\ \mathrm{cal\,mol^{-1}\,Å^{-2}}$).[5] The heat capacity of binding ($\Delta C_p°$) became increasingly negative upon benzo-extension[4]—a change common to "hydrophobic interactions".[6]

We drew two conclusions pertinent to protein–ligand interactions from this study:[4] 1) the balance of enthalpy and entropy responsible for the differences in the partitioning of a ligand, and its benzo-extended analogue, between octanol and buffer is not the same as that responsible for differences in the binding of these ligands to HCA; and 2) the increased binding affinity of the benzo-extended ligands to HCA results from an increased favorability in the enthalpy of binding, and not from an increased entropy of binding. Enthalpy-driven binding of a ligand to HCA is not compatible with the mechanism of the hydrophobic effect proposed by Kauzmann and Tanford (KT),[5,7] but is similar to those observed in other protein–ligand systems in which water is released from the binding pocket upon binding of the ligand.[8–11]

We wished to determine if replacing the four C–H bonds of the benzo moiety with four C–F bonds (i.e., "fluorobenzo-extension") would change the hydrophobic interactions of these ligands with HCA. Fluorocarbons are commonly believed to be "more hydrophobic" than homologous hydrocarbons,[12,13] but typical measures of hydrophobicity—when corrected for differences in surface area—are very similar, if not indistinguishable.[10,13,14] We measured the partitioning of the benzo- and fluorobenzo-extended ligands between buffer and octanol, and found the surface area-corrected hydrophobicity of the ligands increases (by ca. $1.1\ \mathrm{cal\,mol^{-1}\,Å^{-2}}$) upon fluorination (see Supporting Information).

Benzo- and fluorobenzo-extended ligands bind to HCA with similar geometry. Crystal structures of HCA complexed with **F_4BTA**, **H_4BTA**, and **H_8BTA** (Figure 1) show that the binding geometry of these ligands is similar in orientation, despite their differences in shape, volume, and surface. The binding geometry of **F_4BT**, **H_4BT**, and **H_8BT** is also conserved (see Supporting Information).

Careful inspection of the crystal structures of **H_4BTA** and **F_4BTA** reveals that fluorination of the ligand shifts its position in the binding pocket by 0.7 Å (Figure 1D); the positions of the side chains of the amino acids lining the binding pocket of HCA, however, do not change. We attribute this shift of **F_4BTA** to an increased number of unfavorable interactions between the ligand and the binding pocket

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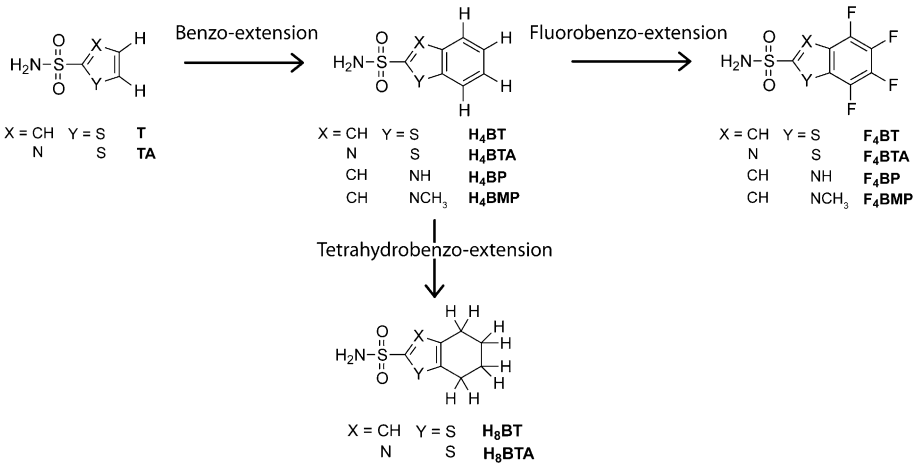
[**] The authors thank Dr. Jasmin Mecinovic, Dr. Ramani Ranatunge, Dr. Demetri Moustakas, Dr. Manza Atkinson, Dr. Mohammad Al-Sayah, Dr. Shuji Fujita, and Mr. Jang Hoon Yoon for their technical contributions. This work was supported by the National Science Foundation (CHE-1152196) and the Wyss Institute of Biologically Inspired Engineering. H.L. thanks the Deutsche Forschungsgemeinschaft (DFG) for a postdoctoral stipend.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201301813.



Scheme 1. Arylsulfonamide ligands. Hydrophobic surface area is added to the heterocyclic ligands by: a "benzo-extension", denoted with an $\mathbf{H_4}$; a "fluorobenzo-extension", denoted with an $\mathbf{F_4}$; or a "tetrahydrobenzo-extension", denoted with an $\mathbf{H_8}$. The bold letters are the ligand acronyms: (**B**)**TA** = (benzo)thiazole, (**B**)**T** = (benzo)thiophene, (**B**)**P** = (benzo)pyrrole, (**B**)**MP** = *N*-methyl-(benzo)pyrrole.

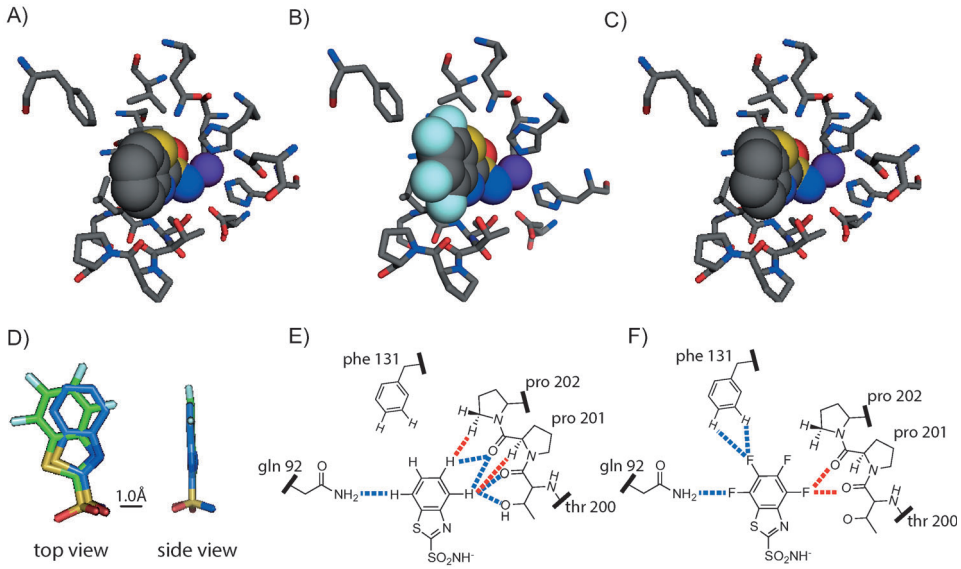


Figure 1. Crystal structures of the active site of HCA complexed with A) **H_4BTA**, B) **F_4BTA**, and C) **H_8BTA**. The purple sphere in each structure represents the Zn^{2+} ion. D) An overlay of the heavy atoms of the **H_4BTA** (blue) and **F_4BTA** (green) ligands from aligned crystal structures. Diagrams of the amino acid residues in contact with the E) benzo-extended portion of **H_4BTA**, and F) the fluorobenzo-extended portion of **F_4BTA**. The dashed lines represent favorable (blue) and unfavorable (red) interactions between the ligand and the protein.

(Figure 1E). The Coulombic repulsion between the fluorine atom on the ligand and the carbonyl of thr 200,[15] a 3.0 Å distance, seems particularly unfavorable.

The atomic composition of the benzo-extension does not affect binding affinity. We measured the enthalpies of binding ($\Delta H°_{bind}$) and the association constants (K_a) for the series of ligands in Scheme 1 using isothermal titration calorimetry (ITC), and estimated the free energies ($\Delta G°_{bind}$) and entropies ($-T\Delta S°_{bind}$) of binding. To account for differences in the pK_a of each ligand, we corrected the measured thermodynamic parameters to represent the binding of the sulfonamide anion to HCA (see Supporting Information).[16]

Remarkably, values of $\Delta G°_{bind}$ of the benzo- and fluorobenzo-extended ligands are indistinguishable at a 90% confidence level (Figure 2A). Values of $\Delta G°_{bind}$, combined with an overall conserved binding geometry of each set of benzo- and fluorobenzo-extended ligands suggest that binding depends on a fine balance of interactions between HCA, the ligand, and molecules of water filling the pocket and surrounding the ligand, and that a simple analysis of interactions between the protein and ligand (Figure 1E) is insufficient to understand (or more importantly, predict) the free energy of binding. Our previous study of **H_4BT** and **H_8BT** showed that changes in the shape of the ligand also resulted in indistinguishable values of $\Delta G°_{bind}$.

The increased binding affinity of **TA** (or **T**) upon benzo- and fluorobenzo-extension is an enthalpy-dominated hydrophobic interaction, and cannot be attributed to the "classical hydrophobic effect" described by KT nor to a "non-classical hydrophobic effect".[17] The partitioning of **H_4BTA** and **F_4BTA** from buffer into octanol (Figure 2B) is, however, an entropy-dominated hydrophobic effect, and in agreement with the KT model.

The release of water from the binding pocket, and not contact between the protein and ligand, affects binding affinity. Comparisons of the crystal structures of **H_4BMP** and **H_4BTA** (or **F_4BMP** and **F_4BTA**, Figure 3) show that the positions of the side chains lining the binding pocket of HCA do not change when the geometry of the bound ligand shifts significantly. The root-mean square deviation (rmsd) for the heavy atoms of the protein in the aligned structures is 0.185 Å for **H_4BMP** and **H_4BTA**, 0.214 Å for **F_4BMP** and **F_4BTA**, and (for comparison) 0.200 Å for **H_4BTA** and **F_4BTA**.

The values of $\Delta G°_{bind}$ of **H_4BMP** and **F_4BMP** are also indistinguishable ($\Delta\Delta G°_{bind} = 0.7 \pm 0.1$ kcal mol^{-1}), and enthalpy-dominated. These results support the hypothesis

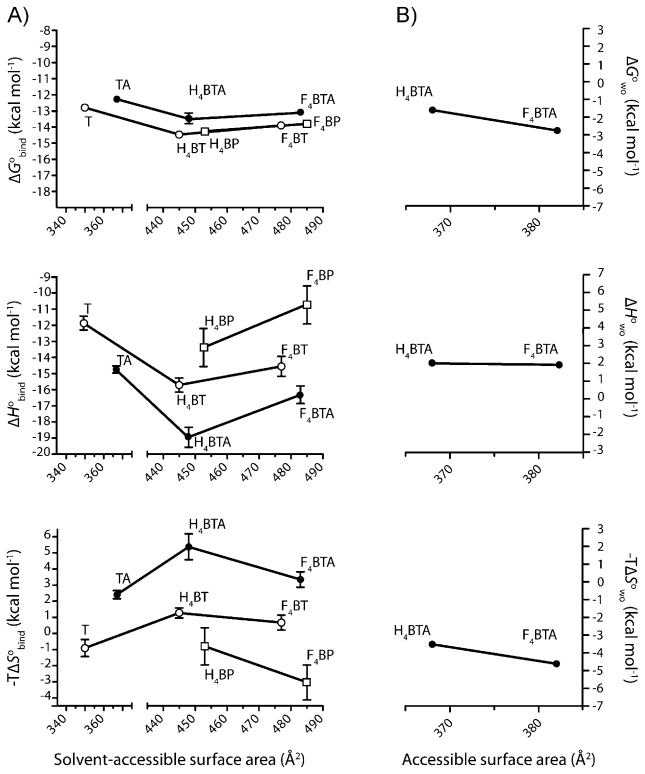


Figure 2. A) Thermodynamics of binding of the anion of each arylsulfonamide ligand to HCA as a function of the difference in solvent-accessible surface area between the bound and unbound states of the ligand. Each datum is the average of at least seven independent measurements, and the error bars represent one standard deviation from the mean. B) Thermodynamics of partitioning of **H_4BTA** and **F_4BTA** from buffer to octanol; each datum is the average of three independent measurements (Supporting Information).

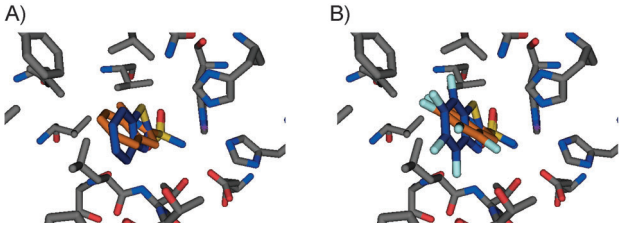


Figure 3. Side-by-side comparison the active site of HCA complexed with (**F**)**BTA** (blue) and (**F**)**BMP** (orange). A) **H_4BTA** and **H_4BMP** and B) **F_4BTA** and **F_4BMP**.

that the increased binding affinity of the benzo-extended ligands is independent of the atomic composition (or molecular properties) of the benzo group.

While the $\Delta G°_{bind}$ is unchanged upon fluorination, we observe significant and compensating changes in $\Delta H°_{bind}$ and $-T\Delta S°_{bind}$ (Figure 2A). To elucidate potential sources of these enthalpy–entropy compensations—a common observation in protein–ligand complexes in which the ligands have very similar structures[18,19]—we calculated the binding energy[20] of **H_4BTA** and **F_4BTA** to HCA, and decomposed these values into the individual energetic components (i.e., Coulombic, van der Waals, desolvation, ligand strain, etc.). The differences between the individual components of the binding energy of **H_4BTA** and **F_4BTA** are indistinguishable (less than 1.5 kcal mol^{-1} different, see Supporting Information) except for: the Coulombic term, which favors the binding of **H_4BTA** by 5.8 kcal mol^{-1}; and the desolvation term, which favors the binding of **F_4BTA** by 8.2 kcal mol^{-1}.

The calculated energies of Coulombic interactions, and the crystal structures of **H_4BTA** and **F_4BTA** (Figure 1E) support the measured values of $\Delta H°_{bind}$ (Figure 2A). The calculated energies of desolvation are consistent with the measured values of $-T\Delta S°_{bind}$, $\Delta G°_{wo}$, and $-T\Delta S°_{wo}$ (Figure 2). We assume the difference in the conformational entropy of the protein–ligand complex is minimal, and thus correlate the desolvation of a ligand as the primary contributor to $-T\Delta S°_{bind}$.[21] The calculated values $\Delta G°_{bind}$ predict that **F_4BTA** will bind to HCA with slightly higher binding affinity than **H_4BTA** (by < 3.0 kcal mol^{-1}), which is within the accuracy limits of the MM-GBSA method.[22] A detailed description of the calculations is presented in the Supporting Information.

Different benzo-extensions cause similar effects on the waters inside the protein pocket. The number of localized (i.e., crystallographically resolvable) waters in the binding pocket of HCA-ligand complexes increases from six to ten when **H_4BTA** is replaced with **F_4BTA** (or four to seven for **H_4BMP** with **F_4BMP**, Table 1). The number of waters localized by the benzo-extended ligands cannot be attributed solely to the solvent-accessible surface area of the ligand (**H_4BTA** 448 Å^2, **F_4BTA** 483 Å^2) because **H_8BTA** (470 Å^2) has a larger surface area than **H_4BTA**, but localizes a smaller number of waters.[4]

We measured values of $\Delta H°_{bind}$ of **TA**, **H_4BTA**, and **F_4BTA** by ITC over a temperature range of 288–307 K, plotted $\Delta H°_{bind}$ as a function of temperature, and estimated the heat capacity of binding ($\Delta C_p°$) for each ligand: **TA** -13 cal mol^{-1}K^{-1}, **H_4BTA** -64 cal mol^{-1}K^{-1}, and **F_4BTA** -108 cal mol^{-1}K^{-1}. The $\Delta C_p°$ of each ligand is negative, and supports our hypothesis of a hydrophobic interaction between the ligands and HCA.[6]

The difference in the heat capacity of **F_4BTA** and **H_4BTA** ($\Delta\Delta C_p° = -44$ cal mol^{-1}K^{-1}) is much larger than the difference calculated from the buried, non-polar surface area of the two ligands (-19 cal mol^{-1}K^{-1}).[6] We attribute this discrepancy between the measured and predicted values of $\Delta C_p°$ to the additional waters observed in the binding pocket of the HCA–**F_4BTA** complex. The value estimated by Connelly for the ordering of a single water (-9 cal mol^{-1}K^{-1})[23] suggests that three additional waters are fixed in the binding pocket of HCA when **H_4BTA** is replaced with **F_4BTA**, and is consistent with the four additional waters observed in the crystal structure.

Increases in binding affinity of ligands correlates with the number of waters released from the binding pocket of HCA, and not with the atomic composition or structure of the ligand. The calorimetry and X-ray crystallography data for the binding of benzo- and fluorobenzo-extended ligands to HCA reinforce our previous conclusion:[4] the hydrophobic effect involved in the binding of arylsulfonamide ligands to HCA is not dominated by a direct interaction between the hydrophobic surfaces of the protein and the ligand, but results

Table 1: Summary of thermodynamic and structural data for the thiazole and methylpyrrole ligands.

Ligand	**H_4BTA**	**F_4BTA**	**H_4BMP**	**F_4BMP**
$\Delta G°_{bind}$ [kcal mol^{-1}]	-13.5 ± 0.4	-13.0 ± 0.2	-13.2 ± 0.1	-13.3 ± 0.1
$\Delta\Delta G°_{bind}$	indistinguishable		indistinguishable	
$\Delta H°_{bind}$ [kcal mol^{-1}]	-18.9 ± 0.5	-16.3 ± 0.6	-12.4 ± 0.5	-8.4 ± 0.6
$-T\Delta S°_{bind}$ [kcal mol^{-1}]	5.5 ± 0.7	3.4 ± 0.5	-0.7 ± 0.5	-4.8 ± 0.7
$\Delta\Delta C_p°$ [cal mol^{-1}][a]	-44		not measured	
Fixed waters[b]	6	10	4	7
ΔGeometry (relative to **H_4BTA**)	–	Translation (0.7 Å)[c]	Rotation (27°)[d]	Rotation (31°)[d]

[a] $\Delta\Delta C_p° = \Delta C_p°$(**$F_4$BTA**) $- \Delta C_p°$(**H_4BTA**). [b] Obtained from crystal structures. [c] Ligand moves in the direction of gln 92. [d] Rotation along the long axis of the ligand.

from waters that are displaced from the binding pocket into the bulk; these waters are less favorable in free energy than waters in the bulk.

The $\Delta G°_{bind}$ of **F_4BTA** and **H_4BTA** to HCA is independent of their exact orientation in the binding pocket, or their molecular structures, as both ligands displace a similar number of waters from the binding pocket. The addition of a benzo-extension to a heterocyclic sulfonamide ligand results in a favorable increase in $\Delta H°_{bind}$; the KT model does not explain the binding of these ligands to HCA, but does explain their partitioning between buffer and octanol. The fluorobenzo extension does result, however, in a decreased favorability of $\Delta H°_{bind}$ and an increased favorability of $-T\Delta S°_{bind}$. We can rationalize the compensation of $\Delta H°_{bind}$ and $-T\Delta S°_{bind}$ in terms of the Coulombic interactions of each ligand with the binding pocket of HCA (i.e., the $\Delta H°_{bind}$ term) and the changes in the energy of solvation (i.e., the $-T\Delta S°_{bind}$ term) of the benzo-extended ligand upon fluorination.

The differences in the thermodynamics of partitioning of these ligands from buffer to octanol, and from buffer to the binding pocket of HCA, support the idea that there is not a single hydrophobic effect reflecting release of water from contacting surfaces of HCA and ligand, but rather aggregated hydrophobic effects that are dependent on the structure of water in the binding pocket of HCA, and on the structure of water surrounding the ligand.

Received: March 3, 2013
Revised: May 10, 2013
Published online: June 20, 2013

Keywords: biomolecular recognition · carbonic anhydrase · hydrophobic effect · protein-ligand binding · water

[1] W. Blokzijl, J. B. F. N. Engberts, *Angew. Chem.* **1993**, *105*, 1610 – 1650; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 1545 – 1579.
[2] N. T. Southall, K. A. Dill, A. D. J. Haymet, *J. Phys. Chem. B* **2002**, *106*, 521 – 533.
[3] P. W. Snyder, M. R. Lockett, D. T. Moustakas, G. M. Whitesides, *Eur. Phys. J. Spec. Top.* **2013**, DOI: 10.1140/epjst/e2013-01818-y.
[4] P. W. Snyder, J. Mecinovic, D. T. Moustakas, S. W. Thomas, M. Harder, E. T. Mack, M. R. Lockett, A. Heroux, W. Sherman, G. M. Whitesides, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17889 – 17894.
[5] C. Tanford, *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 4175 – 4176.
[6] N. V. Prabhu, K. A. Sharp, *Annu. Rev. Phys. Chem.* **2005**, *56*, 521 – 548.
[7] W. Kauzmann, *Annu. Rev. Phys. Chem.* **1957**, *8*, 413 – 438.
[8] W. P. Jencks, *Catalysis in Chemistry and Enzymology*, Dover, New York, **1987**.
[9] S. W. Homans, *Drug Discovery Today* **2007**, *12*, 534 – 539.
[10] J. Mecinović, P. W. Snyder, K. A. Mirica, S. Bai, E. T. Mack, R. L. Kwant, D. T. Moustakas, A. Héroux, G. M. Whitesides, *J. Am. Chem. Soc.* **2011**, *133*, 14017 – 14026.
[11] For a complete listing of references on enthalpy-dominated hydrophobic effects in protein–ligand binding see the Supporting Information.
[12] J. D. Dunitz, *ChemBioChem* **2004**, *5*, 614 – 621.
[13] M. Salwiczek, E. K. Nyakatura, U. I. M. Gerling, S. Ye, B. Koksch, *Chem. Soc. Rev.* **2012**, *41*, 2135 – 2171.
[14] J. C. Biffinger, H. W. Kim, S. G. DiMagno, *ChemBioChem* **2004**, *5*, 622 – 627.
[15] A. Bondi, *J. Phys. Chem.* **1964**, *68*, 441 – 451.
[16] V. M. Krishnamurthy, G. K. Kaufman, A. R. Urbach, I. Gitlin, K. L. Gudiksen, D. B. Weibel, G. M. Whitesides, *Chem. Rev.* **2008**, *108*, 946 – 1051.
[17] E. A. Meyer, R. K. Castellano, F. Diederich, *Angew. Chem.* **2003**, *115*, 1244 – 1287; *Angew. Chem. Int. Ed.* **2003**, *42*, 1210 – 1250.
[18] For a more complete listing of references on enthalpy-entropy compensation in protein–ligand binding see the Supporting Information.
[19] A. Cornish-Bowden, *J. Biosci.* **2002**, *27*, 121 – 126.
[20] P. D. Lyne, M. L. Lamb, J. C. Saeh, *J. Med. Chem.* **2006**, *49*, 4805 – 4808.
[21] E. Freire, *Drug Discovery Today* **2008**, *13*, 869 – 874.
[22] R. Abel, N. K. Salam, J. Shelley, R. Farid, R. A. Friesner, W. Sherman, *ChemMedChem* **2011**, *6*, 1049 – 1066.
[23] P. R. Connelly, *Structure-Based Drug Design: Thermodynamics, Modeling and Strategy*, Springer, Berlin, **1997**.

