Journal of Medicinal Chemistry
Brief Article

J. Med. Chem. **2010,** *53,* 4285–4289 **4285**
DOI: 10.1021/jm901723z

Entropic Contribution to the Linking Coefficient in Fragment Based Drug Design: A Case Study

Valentina Borsi,[†] Vito Calderone,[†] Marco Fragai,[†,‡] Claudio Luchinat,*[,†,‡] and Niko Sarti[†,‡]

[†]*Magnetic Resonance Center (CERM), University of Florence, Via L. Sacconi 6, 50019 Sesto Fiorentino, Italy, and* [‡]*Department of Chemistry, University of Florence, Via Della Lastruccia 3, 50019 Sesto Fiorentino, Italy*

Received November 22, 2009

For several drug leads obtained by tethering weak binding ligands, the dissociation constant is smaller than the product of those of the individual fragments by a factor named the linking coefficient, *E*. This favorable contribution is attributed to the entropic gain that is realized when two weak binding ligands are tethered. Here we show a case study where the linking coefficient is strikingly small ($E = 2.1 \times 10^{-3}$ M^{-1}) and its totally entropic nature is demonstrated.

Introduction

Several high-throughput methods have been developed over the years in order to speed the screening and the identification of new hits for pharmaceutical targets.[1−9] Usually, screening leads to identification of molecules that need subsequent optimization steps to reach a satisfactory affinity. Among the screening methods, the fragment-based approach has seen unquestionable success in the pharmaceutical industry and academic setting because of its high hit rate in generating candidate drugs.[1,9−13]

The leading concept of the fragment-based approach is that a high affinity compound can be designed by tethering with a suitable linker two or more (even weak) ligands able to bind adjacent protein sites.[1] As it is easier to optimize the binding of a small fragment than of a large molecule, linking to separately optimized fragments has a better chance to produce compounds with nanomolar dissociation constant K_D. As an added value, in several cases the K_D of the tethered molecules is found to be smaller than the product of the K_D values of the single fragments.[14,15] This additional increase in affinity can be explained by considering that when two ligands bind to a protein, each of them loses a fraction of rigid body rotational and translational entropy. Conversely, when the two ligands are tethered in a single molecule, only one unfavorable rigid body entropy barrier affects the binding.[16] This smaller loss of entropy (i.e., relative entropic gain) accounts for the linking effect.[17,18] For a two-fragment A−B molecule, the dissociation constant K_D^{AB} is often reported as the product of the dissociation constants of the isolated fragments A and B, K_D^{A} and K_D^{B}, multiplied by a term named linking coefficient, *E*:[1,19]

$$K_D^{AB} = K_D^{A} K_D^{B} E \quad (\text{or equivalently, } \Delta G^{AB} = \Delta G^{A} + \Delta G^{B} + RT \ln E) \tag{1}$$

where $E < 1$ M^{-1} implies a favorable contribution to binding. In an ideal case, the linking coefficient *E*, obtained from eq 1 when the three binding constants are known from experiments, would thus be a direct measure of the entropic gain.

However, besides the favorable entropic contribution, there may be unfavorable enthalpic contributions to the binding that arise from distortions of the target binding geometry of the two fragments when linked together, as well as favorable or unfavorable interactions of the linker itself with the target molecule. Indeed, it has been observed that the linking coefficient is strongly dependent on the structural features of the tethered molecule and on its binding mode to the target. In some cases linking coefficients even larger than 1 (i.e., unfavorable) have been found, while in successful cases linking coefficient values slightly smaller than 10^{-2} M^{-1} have been observed.[15] The most favorable *E* (3.1×10^{-3} M^{-1}) has been observed for a biphenyl-based inhibitor of matrix metalloproteinase 2 (MMP-2).[15] Although the pure entropic term would be theoretically capable of accounting by itself for linking coefficients as low as 10^{-8} M^{-1},[16] experimental verification that even *E* values of the order of 10^{-2} M^{-1} are entirely due to a favorable entropic term is still lacking.

One way to obtain this verification would be to construct a two-fragment molecule where the two fragments are able to bind separately to the target in such a way that they just need to be linked together by one covalent bond without any intervening atom. The increase in binding affinity of a zero-length tether molecule of this type should be, at least to a first approximation, entirely due to the entropic effect, provided that the enthalpic contributions are essentially unchanged; i.e., the two fragments maintain all relevant interactions with the target when bound together. Interestingly, at least one case of this type has been described, and a reasonable although not extremely small *E* of about 3.3×10^{-2} M^{-1} has been reported.[20] Unfortunately, experimental verification of its entropic nature through, for example, calorimetry is not available.

Here we report on a sulfonamide derivative inhibitor of matrix metalloproteinases (MMP[a]) whose components have been designed by application of a *deconstructing* procedure,[21] and where the linker is just one covalent bond. Sulfonamide

*To whom correspondence should be addressed. Address: Magnetic Resonance Center (CERM), University of Florence, Via L. Sacconi 6, 50019 Sesto Fiorentino, Italy. Phone: +39055 4574262. Fax: +39055 4574253. E-mail: luchinat@cerm.unifi.it.

[a] Abbreviations: MMP, matrix metalloproteinase; PMAHA, *N*-hydroxy-2-(4-methoxyphenylsulfonamido)acetamide; PMS, paramethoxybenzenesulfonylamide; AHA, acetohydroxamic acid; ITC, isothermal titration microcalorimetry.

Published on Web 04/23/2010

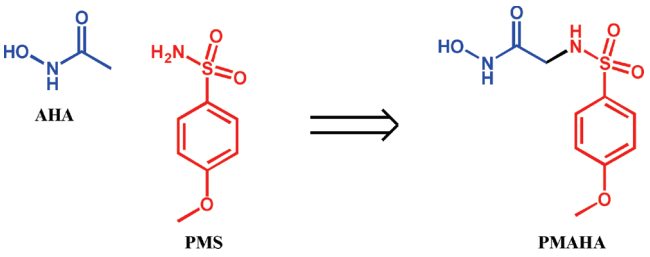

Figure 1. *N*-Hydroxy-2-(4-methoxyphenylsulfonamido)acetamide (PMAHA) can be considered as formed by two fragments, paramethoxybenzenesulfonylamide (PMS) and acetohydroxamic acid (AHA).

derivatives constitute one of the most important classes of inhibitors of zinc enzymes, possessing a high affinity toward several zinc enzyme families including, besides MMPs, carbonic anhydrases.[22−27] In the case of MMPs, sulfonamide inhibitors consist of a zinc binding group such as hydroxamate or carboxylate and of a lipophilic moiety interacting with the S1′ cavity.[28] *N*-Hydroxy-2-(4-methoxyphenylsulfonamido)acetamide (PMAHA, Figure 1) is a simple but relatively potent inhibitor of MMP-12.[29,30] Virtually, this molecule can be considered as formed by two fragments, paramethoxybenzenesulfonylamide (PMS) and acetohydroxamic acid (AHA) (Figure 1), tethered by a simple covalent bond upon removal of one hydrogen atom on each side. The present case study analyzes the binding of PMAHA and its constituting fragments PMS and AHA to the catalytic domain of MMP-12. The system displays a very small E of 2.1×10^{-3} M^{-1}, and it is shown that this value is entirely due to a very favorable entropic contribution, the enthalpic term being very similar to the sum of those of the two isolated fragments.

Experimental Section

The studies were performed on the F171D mutant of human MMP-12 catalytic domain, corresponding to the stretch Gly106−Gly263. The protein was expressed as inclusion body, purified by FPLC and refolded as previously reported.[29]

Isothermal titration microcalorimetry experiments were performed at 298 K with a VP-ITC microcalorimeter (MicroCal, Inc., Northampton, MA). After an initial injection of 1 μL, aliquots of 10 μL of 200 μM inhibitor (BSAHA) with 0.1% (v/v) DMSO were stepwise injected into the sample cell containing a 20 μM solution of MMP12 catalytic domain until complete saturation was obtained. All experiments were performed in 20 mM Tris (pH 7.2), 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 0.3 M NaCl, 4 mM AHA, with PMS concentrations ranging from 0.1 to 3 mM. Heats of dilution were measured by injecting the ligand solution into buffer, and the obtained values were subtracted from the binding heats. The thermodynamic parameters and K_A values were calculated by fitting the data to a single binding site model with the Origin 7.0 sofware (Microcal, Inc.).

Binding of PMS to the ^{15}N enriched catalytic domain of MMP-12 was monitored by $^{1}H-^{15}N$ heteronuclear single quantum coherence (HSQC) NMR spectroscopy (see Supporting Information).

Crystals of human MMP12, already containing AHA from the refolding process, grew at 20 °C from a 0.1 M Tris-HCl, 30% PEG 6000, 200 mM AHA, 1.0 M $LiCl_2$ solution at pH 8.0 using the vapor diffusion technique. The final protein concentration was about 10 mg/mL. A soaking procedure was carried out to obtain the adduct with PMS (see Supporting Information).

Results

The interaction of PMS with the active site of MMP-12 was analyzed by X-ray crystallography, NMR, and microcalorimetry. The dissociation constant of PMS was obtained from the analysis of the chemical shift perturbations on the assigned $^{1}H-^{15}N$ HSQC spectra of the free and AHA-inhibited form of MMP-12. The dissociation constant (K_D = 1.5×10^{-3} M) was not affected by the presence of AHA in solution. The binding mode of the ligand in the active site of the protein was investigated by soaking crystals of MMP-12, incorporating the weak AHA inhibitor, in solutions of PMS.

In the X-ray structure, the PMS ligand (PDB code 3LKA) fits the S1′ cavity with the *p*-methoxybenzene group and establishes an H-bond between one of its sulfonyl oxygens (O2) and the amide nitrogen of Leu181 (2.93 Å) (Figure 2B). The sulfonamide nitrogen is 4.2 Å from the catalytic zinc (the same distance is 4.9 Å in the PMAHA complex), and any interaction with the metal ion can be excluded. At the same time, the free AHA ligand chelates the zinc ion with its two oxygens, and the interaction is further stabilized by two H-bonds, one between the protonated oxygen atom (O4) of AHA and the carboxylate Oδ2 of Glu219 (2.53 Å) and the second (2.76 Å) between the hydroxamate NH and the carbonyl oxygen of Ala182. The two ligands bind independently to the protein and do not interact with one another. Moreover, the AHA ligand exhibits the same binding mode in the presence and in the absence[29] of PMS. The analysis of crystallographic data (PDB code 3LKA) revealed the presence of a water molecule (HOH 2) between PMS and AHA, 2.63 Å from the zinc ion with an occupancy of 0.40 ± 0.2. Also in the PDB file 1Y93, where AHA alone binds in the active site of MMP-12, a water molecule (HOH 408) with low occupancy (0.30 ± 0.1) is present 2.65 Å from the zinc ion. Such low occupancies allow one to postulate that the water molecules facing the hydroxamic acid are indeed labile and not present in the majority of protein molecules of these adducts. While a modest entropic gain may still be present because of the absence of this water molecule in complex with PMAHA, this gain is probably much smaller than 1 kcal/mol,[31] and therefore, the main finding of the present work is unchanged.

The binding of PMAHA (Figure 2C) and AHA alone (Figure 2A) with MMP-12 was characterized previously by X-ray, microcalorimetry, and enzymatic assay.[29,30] In the complex of MMP-12 with PMAHA (PDB code 3LK8), the inhibitor binds the catalytic zinc ion by its hydroxamic moiety, and its *p*-methoxybenzene group fits the S1′ cavity.

The paramethoxysulfonyl moiety in the PMAHA molecule shares the same binding mode of the isolated PMS ligand, while the AHA moiety appears rotated by ~45° with respect to the position of the isolated AHA ligand.[29] Despite this rotation, all the interactions observed for the two fragments are in place: the bidentate behavior of the AHA moiety, the sulfonyl oxygen at 2.93 Å from the amide nitrogen of Leu181, the protonated oxygen atom (O4) at 2.53 Å from the carboxylate Oδ2 of Glu219, and the hydroxamate NH at 2.76 Å from the Ala182 carbonyl oxygen. A series of ITC measurements on the binding of a parent arysulfonamide inhibitor (BSAHA, see Figure 1S in Supporting Information) were performed in the presence of various PMS concentrations in the range 0.1−3 mM. AHA was also present in solution at 4 mM to prevent the self-hydrolysis of the protein. By fitting the obtained enthalpy as a function of PMS concentration and after correction for the competition with AHA (see Supporting Information), a ΔH^0 of −6.0 kcal/mol was found for the binding of PMS. The ΔG^0, ΔH^0, ΔS^0, and K_D for the binding

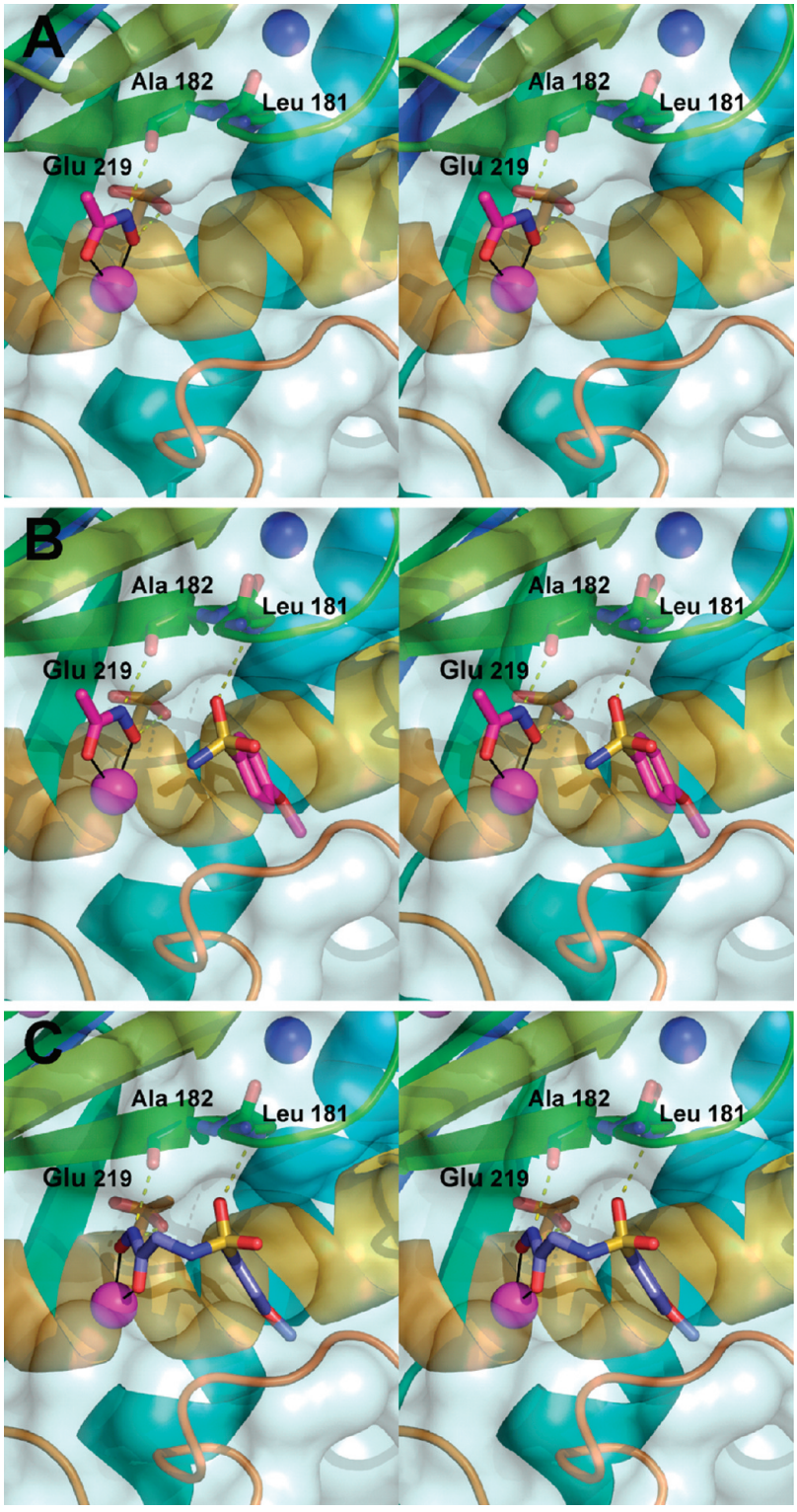

Figure 2. Stereoview of the active site of MMP-12 complexed with hydroxamic acid (A). PMS and AHA bind independently to the active site of MMP-12 and do not interact with one another (B). The presence of PMS does not alter the binding mode of AHA. The tethered molecule PMAHA maintains all the interactions observed for the two isolated fragments (C). Only the AHA moiety appears rotated with respect to the position of the isolated AHA ligand but with all relevant protein interactions maintained.

of AHA and PMAHA to MMP-12 at 298 K were determined previously[30] and are reported in Table 1. For PMS the free energy of binding (ΔG^0) was calculated from the dissociation constant obtained by NMR. The ΔS^0 was estimated from the relationship $\Delta G^0 = \Delta H^0 - T\Delta S^0$. The ΔH_0 for PMS was determined by isothermal titration microcalorimetry (ITC) in this work. As shown in Table 1, the ΔH^0 found for PMAHA (-8.52 kcal/mol) is similar to the sum of the binding enthalpy of AHA and PMS. At the same time the free energy of binding for PMAHA is much more negative than the sum of the ΔG^0 of the isolated fragments. The resulting linking coefficient is about 2.1×10^{-3} M^{-1} (see Table 1).

Table 1. Corrected Thermodynamic Parameters for the Investigated Inhibitors

	K_D (M)	ΔG^0 (kcal/mol)	ΔH^0 (kcal/mol)	$-T\Delta S^0$ (kcal/mol)
AHA	6.2×10^{-3}	−3.01	−3.18	+0.17
PMS	1.5×10^{-3}	−3.85	−6.00	+2.15
PMAHA	2.0×10^{-8}	−10.50	−8.52	−1.98
AHA + PMS		−6.86	−9.18	+2.32
ΔΔ(kcal/mol)		−3.64	+0.66	−4.30

Discussion

The large affinity observed for PMAHA is in line with expectations for this class of MMP inhibitors. The deconstructing analysis of this simple linked molecule provides a new piece of evidence to rationalize the key factors playing a role in the tethering approach.

Although theoretical models predict, for the dimerization of adjacent ligands, affinity advantages up to 10^{-8} M with respect to either fragment, the experience on real cases shows that usually the gain in affinity is orders of magnitude smaller.[16,32] In fact, the linkage of the fragments often results in a new molecule that does not allow each of them to adopt the original and optimal binding mode.[21]

In this respect, PMAHA with the related fragments PMS and AHA is a good case study because all the requirements relevant to maximize the linking effect are satisfied in the molecule. In particular, the structural data show that the binding mode of the two individual fragments is substantially maintained in the joined molecule. Moreover, no sizable additional favorable interactions or unfavorable strain energies are introduced by the linker, since the two fragments are "tethered" by a single covalent bond without any additional intervening atom.

Hydroxamic inhibitors are well-known to bind MMPs (but MMP-3) without the transfer of protons.[33,34] The peculiar pH dependence of the binding affinity observed in MMP-3 for hydroxamic inhibitors is accounted for by the protonation of His224,[35] which is not present in other MMPs. In MMP-12 residue 224 is a lysine, which is definitely protonated up to at least pH 10. Concerning the catalytically relevant Glu219, a peculiar feature of MMPs is that the Glu219-hydroxamic acid moiety maintains a total of one of the two ionizable protons when the complex is formed over a broad pH range between 5 and 9.[33] At the pH of the present experiments Glu 219 is deprotonated and the hydroxamic acid is protonated so that the total number of ionizable protons does not change upon binding. Actually, the proton forming the H-bond between Glu219 and the zinc coordinated water molecule is replaced in the complex by the hydrogen of the hydroxamic moiety. No other titratable groups are present in the active site of MMP-12. Therefore, no protonation or deprotonation reactions occur at pH 7.2 for the ligands PMAHA, BMAHA, and AHA studied in this work. As far as PMS is concerned, proton transfer to or from the enzyme−inhibitor complex during binding is unlikely also for this ligand, whose amide group should have a pK_a above 10 (a pK_a of 10.3 was calculated by the ACD/Labs software). To further check that the binding of PMS does not involve protonation/deprotonation reactions, the pH dependence of its K_i was studied by fluorimetric assays (see Supporting Information) at pH 6.7, 7.2, and 8. The K_i is independent of pH over the range 6.7−8.0 where it is $(3 \pm 1) \times 10^{-3}$ M. Collectively, all these data provide some support to the hypothesis that the enthalpy of binding of PMS is not affected by proton transfer reactions.

As already shown in Table 1, PMAHA exhibits a dissociation constant (K_D^{AB}) of 2.0×10^{-8} M, much lower than the dissociation constants of the two fragments PMS ($K_D^A = 1.5 \times 10^{-3}$ M) and AHA ($K_D^B = 6.2 \times 10^{-3}$ M) and nearly 3 orders of magnitude lower than the product of their relative affinity constants.

A suitable approach to investigate the linking coefficient is to compare the difference in experimental ΔG^0 and ΔH^0 values and in the calculated entropy contributions. For PMAHA the difference in experimental ΔG^0 ($\Delta\Delta G^0$) with respect to the sum of the ΔG^0 values for the isolated PMS and AHA is large and negative (−3.64 kcal/mol). This increased affinity is not related to an improved interaction, as shown by the small (and even positive) difference (+0.66 kcal/mol) in experimental ΔH^0 ($\Delta\Delta H^0$) between PMAHA and the isolated PMS and AHA. On the contrary, the difference in $T\Delta S^0$ for PMAHA is high and negative (−4.30 kcal/mol) and fully accounts for the large linking effect observed for the tethered molecule.

Besides the calculation of the value for the linking coefficient for the investigated molecule, the present analysis allows to us to achieve a more general view on the entropic contribution in fragment-based drug design. In particular, in the case of PMAHA no additional enthalpic and entropic terms arising from atoms of the linker contribute to ΔH^0 and to ΔS^0. At the same time, other possible contributions to ΔS^0 are probably negligible, as suggested by the superimposition of the complexes and by the small difference in the interaction energy ($\Delta\Delta H^0 = -0.66$ kcal/mol). Collectively, all these findings provide an estimation of the value for the linking coefficient that can be reasonably achieved by tethering two ligands when the interactions of each of them are maintained and no additional effects arise from the atoms of the linker. In this respect, for PMAHA an increase in free energy of binding even larger than 4.30 kcal/mol would have been possible without the small unfavorable enthalpic term of +0.66 kcal/mol, possibly related to small changes in binding mode. At the same time, the observed affinity can hardly be due to an increase in hydrophobicity of the tethered molecule with respect to the two fragments (see Supporting Information).

This large value also opens interesting questions on the advantages provided by the linking coefficient in fragment-linking and fragment growing strategies.[36] In the former, additional atoms are introduced to tether the fragments without altering the original binding mode. Obviously the length and the structure of the linker are designed to avoid steric clashes, to reduce the degrees of freedom, and possibly to establish interactions with the target. However, the linker is not conceived to maximize these interactions, and detrimental enthalpic and entropic contributions to the free energy of binding can drastically reduce the benefit of the linking coefficient.[37] On the contrary, in the fragment growing approach at each step the additional moiety introduced in the scaffold is conceived to establish new strong interactions with the target without altering the optimal binding mode of the original core. Therefore, the latter strategy might theoretically take the best advantage from the entropic gain related to the linking coefficient. However, in real cases, several factors such as the lack of detailed structural information and the protein flexibility make it difficult to obtain suitable growth of the fragment, and the two strategies usually result in ligands with similar affinity constants.[38]

In summary we have shown that when the two fragments are tethered with a zero-length linker in a way that all the main interactions are maintained, linking coefficient values

approaching 10^{-3} M^{-1} can be obtained. We have also verified the entropic nature of the linking coefficient, which in the absence of unfavorable enthalpic contribution can be even smaller than 10^{-3} M^{-1}.

Acknowledgment. This work was supported by EC (Grants MEST-CT-2004-504391, SFMET No. 201640, SPINE2-COMPLEXES No. 031220, EPISODE No. 229761, INSTRUCT No. 211252, and e-NMR No. 213010), by MIUR (Grants PRIN 2005, Prot. N. 2005039878, Prot. RBLA032ZM7, Prot. RBIP06LSS2), and by Ente Cassa di Risparmio di Firenze.

Supporting Information Available: Crystallization, data collection and refinement statistics for MMP-12-PMS complex, calculated partition coefficients, details of ITC measurements, NMR analysis and fluorimetric assays. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. Discovering high-affinity ligands for proteins: SAR by NMR. *Science* **1996**, *274*, 1531–1534.
(2) Erlanson, D. A.; Braisted, A. C.; Raphael, D. R.; Randal, M.; Stroud, R. M.; Gordon, E. M.; Wells, J. A. Site-directed ligand discovery. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9367–9372.
(3) Bajorath, F. Integration of virtual and high-throughput screening. *Nat. Rev. Drug Discovery* **2002**, *1*, 882–894.
(4) Bleicher, K. H.; Bohm, H. J.; Muller, K.; Alanine, A. I. Hit and lead generation: beyond high-throughput screening. *Nat. Rev. Drug Discovery* **2003**, *2*, 369–378.
(5) Blundell, T. L.; Jhoti, H.; Abell, C. High-throughput crystallography for lead discovery in drug design. *Nat. Rev. Drug Discovery* **2002**, *1*, 45–54.
(6) Congreve, M.; Carr, R.; Murray, C.; Jhoti, H. A "rule of three" for fragment-based lead discovery? *Drug Discovery Today* **2003**, *8*, 876–877.
(7) Bertini, I.; Fragai, M.; Lee, Y.-M.; Luchinat, C.; Terni, B. Paramagnetic metal ions in ligand screening: the Co^{II} matrix metalloproteinase 12. *Angew. Chem., Int. Ed.* **2004**, *43*, 2254–2256.
(8) Bertini, I.; Fragai, M.; Luchinat, C.; Talluri, E. Water-based ligand screening for paramagnetic metalloproteins. *Angew. Chem., Int. Ed.* **2008**, *47*, 4533–4537.
(9) Pellecchia, M.; Bertini, I.; Cowburn, D.; Dalvit, C.; Giralt, E.; Jahnke, W.; James, T. L.; Homans, S. W.; Kessler, H.; Luchinat, C.; Meyer, B.; Oschkinat, H.; Peng, J.; Schwalbe, H.; Siegal, G. Perspectives on NMR in drug discovery: a technique comes of age. *Nat. Rev. Drug Discovery* **2008**, *7*, 738–745.
(10) Hajduk, P. J.; Greer, J. A decade of fragment-based drug design: strategic advances and lessons learned. *Nat. Rev. Drug Discovery* **2007**, *6*, 211–219.
(11) Chen, Y.; Shoichet, B. K. Molecular docking and ligand specificity in fragment-based inhibitor discovery. *Nat. Chem. Biol.* **2009**, *5*, 358–364.
(12) Ciulli, A.; Williams, G.; Smith, A. G.; Blundell, T. L.; Abell, C. Probing hot spots at protein−ligand binding sites: a fragment-based approach using biophysical methods. *J. Med. Chem.* **2006**, *49*, 4992–5000.
(13) Pellecchia, M. Fragment-based drug discovery takes a virtual turn. *Nat. Chem. Biol.* **2009**, *5*, 274–275.
(14) Hajduk, P. J.; Sheppard, G.; Nettesheim, D. G.; Olejniczak, E. T.; Shuker, S. B.; Meadows, R. P.; Steinman, D. H.; Carrera, G. M.; Marcotte, P. A.; Severin, J.; Walter, K.; Smith, H.; Gubbins, E.; Simmer, R.; Holzman, T. F.; Morgan, D. W.; Davidsen, S. K.; Summers, J. B.; Fesik, S. W. Discovery of potent nonpeptide inhibitors of stromelysin using SAR by NMR. *J. Am. Chem. Soc.* **1997**, *119*, 5818–5827.
(15) Olejniczak, E. T.; Hajduk, P. J.; Marcotte, P. A.; Nettesheim, D. G.; Meadows, R. P.; Edalji, R.; Holzman, T. F.; Fesik, S. W. Stromelysin inhibitors designed from weakly bound fragments: effects of linking and cooperativity. *J. Am. Chem. Soc.* **1997**, *119*, 5828–5832.
(16) Page, M. I.; Jencks, W. P. Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678–1683.
(17) Jencks, W. P. On the attribution and additivity of binding energies. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4046–4050.
(18) Murray, C. W.; Verdonk, M. L. The consequences of translational and rotational entropy lost by small molecules on binding to proteins. *J. Comput.-Aided Mol. Des.* **2002**, *16*, 741–753.
(19) Pellecchia, M. Solution nuclear magnetic resonance spectroscopy techniques for probing intermolecular interactions. *Chem. Biol.* **2005**, *12*, 961–971.
(20) Congreve, M. S.; Davis, D. J.; Devine, L.; Granata, C.; O'Reilly, M.; Wyatt, P. G.; Jhoti, H. Detection of ligands from a dynamic combinatorial library by X-ray crystallography. *Angew. Chem., Int. Ed.* **2003**, *42*, 4479–4482.
(21) Babaoglu, K.; Shoichet, B. K. Deconstructing fragment-based inhibitor discovery. *Nat. Chem. Biol.* **2006**, *2*, 720–723.
(22) MacPherson, L. J.; Bayburt, E. K.; Capparelli, M. P.; Carroll, B. J.; Goldstein, R.; Justice, M. R.; Zhu, L.; Hu, S.; Melton, R. A.; Fryer, L.; Goldberg, R. L.; Doughty, J. R.; Spirito, S.; Blancuzzi, V.; Wilson, D.; O'Byrne, E. M.; Ganu, V.; Parker, D. T. Discovery of CGS 27023A, a non-peptidic, potent, and orally active stromelysin inhibitor that blocks cartilage degradation in rabbits. *J. Med. Chem.* **1997**, *40*, 2525–2532.
(23) Bertini, I.; Fragai, M.; Luchinat, C. Intra- and interdomain flexibility in matrix metalloproteinases: functional aspects and drug design. *Curr. Pharm. Des.* **2009**, *15*, 3592–3605.
(24) Stockmann, H.; Bronowska, A.; Syme, N. R.; Thompson, G. S.; Kalverda, A. P.; Warriner, S. L.; Homans, S. W. Residual ligand entropy in the binding of p-substituted benzenesulfonamide ligands to bovine carbonic anhydrase II. *J. Am. Chem. Soc.* **2008**, *130*, 12420–12426.
(25) Whitesides, G. M.; Krishnamurthy, V. M. Designing ligands to bind proteins. *Q. Rev. Biophys.* **2005**, *38*, 385–395.
(26) Srivastava, D. K.; Jude, K. M.; Banerjee, A. L.; Haldar, M.; Manokaran, S.; Kooren, J.; Mallik, S.; Christianson, D. W. Structural analysis of charge discrimination in the binding of inhibitors to human carbonic anhydrases I and II. *J. Am. Chem. Soc.* **2007**, *129*, 5528–5537.
(27) Matulis, D.; Todd, M. In *Thermodynamics/Structure Correlations of Sulfonamide Inhibitor Binding to Carbonic Anhydrase in Biocalorimetry 2*; Ladbury, J., Doyle, M., Eds.; Wiley & Sons: New York, 2004.
(28) Bertini, I.; Calderone, V.; Fragai, M.; Luchinat, C.; Mangani, S.; Terni, B. Crystal structure of the catalytic domain of human matrix metyalloproteinase 10. *J. Mol. Biol.* **2004**, *336*, 707–716.
(29) Bertini, I.; Calderone, V.; Cosenza, M.; Fragai, M.; Lee, Y.-M.; Luchinat, C.; Mangani, S.; Terni, B.; Turano, P. Conformational variability of MMPs: beyond a single 3D structure. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5334–5339.
(30) Bertini, I.; Calderone, V.; Fragai, M.; Giachetti, A.; Loconte, M.; Luchinat, C.; Maletta, M.; Nativi, C.; Yeo, K. J. Exploring the subtleties of drug-receptor interactions: the case of matrix metalloproteinases. *J. Am. Chem. Soc.* **2007**, *129*, 2466–2475.
(31) Dunitz, J. D. The entropic cost of bound water in crystals and biomolecules. *Science* **1994**, *264*, 670.
(32) Whitty, A. Cooperativity and biological complexity. *Nat. Chem. Biol.* **2008**, *4*, 435–439.
(33) Cha, J.; Auld, D. S. Site-directed mutagenesis of the active site glutamate in human matrilysin: investigation of its role in catalysis. *Biochemistry* **1997**, *36*, 16019–16024.
(34) Parker, M. H.; Lunney, E. A.; Ortwine, D. F.; Pavlovsky, A. G.; Humblet, C.; Brouillette, C. G. Analysis of the binding of hydroxamic acid and carboxylic acid inhibitors to the stromelysin-1 (matrix metalloproteinase-3) catalytic domain by isothermal titration calorimetry. *Biochemistry* **1999**, *38*, 13592–13601.
(35) Holman, C. M.; Kan, C. C.; Gehring, M. R.; Van Wart, H. E. Role of His-224 in the anomalous pH dependence of human stromelysin-1. *Biochemistry* **1999**, *38*, 677–681.
(36) Ciulli, A.; Abell, C. Fragment-based approaches to enzyme inhibition. *Curr. Opin. Biotechnol.* **2007**, *18*, 489–496.
(37) Chung, S.; Parker, J. B.; Bianchet, M.; Amzel, L. M.; Stivers, J. T. Impact of linker strain and flexibility in the design of a fragment-based inhibitor. *Nat. Chem. Biol.* **2009**, *5*, 407–413.
(38) Hung, A. W.; Silvestre, H. L.; Wen, S.; Ciulli, A.; Blundell, T. L.; Abell, C. Application of fragment growing and fragment linking to the discovery of inhibitors of *Mycobacterium tuberculosis* pantothenate synthetase. *Angew. Chem., Int. Ed.* **2009**, 8452–8456.