CHEMBIOCHEM

DOI: 10.1002/cbic.200500089

An Experimental and Molecular-Modeling Study of the Binding of Linked Sulfated Tetracyclitols to FGF-1 and FGF-2

Siska Cochran, Cai Ping Li, and Ian Bytheway*[a]

The experimental binding affinities of a series of linked sulfated tetracyclitols [Cyc_2N-R-NCyc_2, where $Cyc = C_6H_6(OSO_3Na)_3$ and R = $(CH_2)_n$ (n=2–10), p-xylyl or $(C_2H_4)_2$-Ncyc] for the fibroblast growth factors FGF-1 and FGF-2 have been measured by using a surface plasmon resonance assay. The K_D values range from 7.0 nM to 1.1 μM for the alkyl-linked ligands. The binding affinity is independent of the flexibility of the linker, as replacement of the alkyl linker with a rigid p-xylyl group did not affect the K_D. Calculations suggest that binding modes for the p-xylyl-linked ligand are similar to those calculated for the flexible alkyl-linked tetracyclitols. The possible formation of cross-linked FGF:cyclitol complexes was examined by determining K_D values at increasing protein concentrations. No changes in K_D were observed; this suggesting that only 1:1 complexes are formed under these assay conditions. Monte Carlo multiple-minima calculations of low-energy conformers of the FGF-bound ligands showed that all of the sulfated tetracyclitol ligands can bind effectively in the heparan sulfate-binding sites of FGF-1 and FGF-2. Binding affinities of these complexes were estimated by the Linear Interaction Energy (LIE) method to within a root-mean-square deviation of 1 kcal mol^{-1} of the observed values. The effect of incorporating cations to balance the overall charge of the complexes during the LIE calculations was also explored.

Introduction

Tumor cells require a blood supply for growth and metastasis. The involvement of the fibroblast growth factors (FGFs) in tumour angiogenesis, the sprouting of new blood vessels from pre-existing vessels surrounding the tumour, has made these proteins an attractive target for cancer therapy.[1,2] FGFs interact with either soluble or cell-surface-bound heparan sulfate (HS) to promote FGF-receptor dimerization, activation and subsequent initiation of an intracellular response.[3,4] Inhibition of this response by ligands able to compete efficiently with HS for FGFs is, therefore, a promising strategy for the prevention or mitigation of tumour-induced angiogenesis.

A variety of HS mimics have been explored with the aim of blocking FGF:HS binding. Dipeptide derivatives[5] and sulfated glycoconjugates[6] are known to bind to FGFs, whereas synthetic HS-derived oligosaccharides,[7,8] poly(*N*-acryl amino acids),[9] sulfonic acid polymers,[10] hydroxyl- and carboxylated polyaromatic compounds,[11] oligoribonucleic acids[12] and derivatives of glucuronic acids[13] all compete with HS to block cell signaling and proliferation in vitro. Similarly, the antiangiogenic activity of sulfated polysaccharides and oligosaccharides,[14–16] is attributed to the binding of these ligands to the HS-binding sites of the FGF proteins.

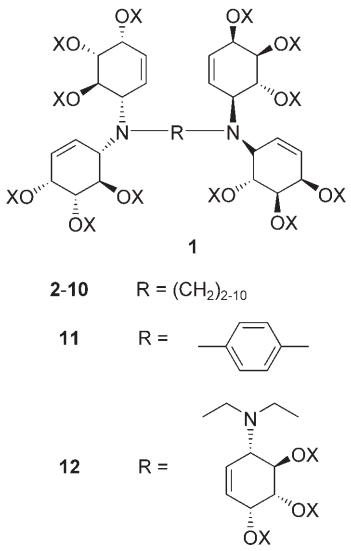
The interest in HS mimics has lead to the use of several compounds in clinical trials for the treatment of cancer. One example is PI-88,[17,18] which binds with high affinity to the FGF proteins[19,20] and competes with HS to block cell signaling and proliferation in vitro.[21] Another well-characterized inhibitor that has undergone clinical trials is suramin, a polysulfonated binaphthyl urea[22] that inhibits FGF-2-induced angiogenesis. The related suradistas, derivatives of synthetic binaphthalene sulfonic distamycin A, have also been tested in preclinical studies.[23,24]

Smaller molecules can also bind to HS-binding sites. NMR studies of FGF-1 complexed with 1,3,6-naphthalene trisulfonate, the functional component of the suramins and suradistas, showed that this ligand is stabilized in the HS-binding site of FGF-1.[25] More recently, several new naphthalene sulfonate derivatives were tested for their ability to inhibit the mitogenic activity of FGF-1, and a crystal structure of FGF-1 complexed to the most active of these has provided clues to the functional significance of the different substituents on the ligand.[26]

In this study we have measured the binding affinities of a series of sulfated linked tetracyclitols[27] (**1**, Scheme 1) for both FGF-1 and FGF-2. These ligands are characterized by two amino-dicyclitol groups ($NCyc_2$ where $Cyc = C_6H_6(OSO_3Na)_3$) joined by a linker composed of either alkyl chains (**2**–**10**, Scheme 1) or other functional groups (**11**–**12**, Scheme 1). The ability of these cyclitol ligands to inhibit a variety of heparin- and HS-binding proteins has been demonstrated, and the HS-mimetic nature of these ligands has been established.[28] This work focuses on the nature of the interactions between these

[a] *Dr. S. Cochran, Dr. C. P. Li, Dr. I. Bytheway*
Drug Design Group, Progen Industries Ltd.
P.O. Box 28, Richlands BC, Queensland 4077 (Australia)
Fax: (+617) 3375-6746
E-mail: ian.bytheway@progen.com.au

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.



Scheme 1. Structures of the linked sulfated tetracyclitols where X denotes SO_3Na.

ligands and the growth factors FGF-1 and FGF-2 through examination of binding affinities obtained from a solution-affinity assay. These binding affinities were rationalized by using a combination of conformational search and linear interaction energy (LIE) calculations.

Results and Discussion

Ligand-binding studies of 2–10

The binding affinities of **2**–**10** for FGF-1 and FGF-2 were measured by using a solution affinity assay in which free ligand competes with immobilized heparin for protein. The strengths of the FGF:**1** interactions were measured by surface plasmon resonance (SPR) spectroscopy.[20] An important aspect of this assay is its specificity as FGF–ligand binding is only detected when the heparin-binding site is involved. Thus, nonspecific binding to other sites of the protein is not evaluated. Examples of typical sensorgrams and fitting plots for **6** binding to both FGF-1 and FGF-2 are given in Figure 1.

The K_D values for **2**–**10** binding to FGF-1 and FGF-2 range from 7.2 nM to 1.1 μM (Table 1). Thus, these tetracyclitols bind to FGF-1 as strongly as both heparin (2.4 nM) and low-molecular-weight heparin (LMWH, 17 nM).[20] Their affinities for FGF-2[20] are similar to that observed for LMWH (86 nM) but lower than that of heparin (5 nM).

The observed FGF-2-binding affinities of **1** are consistently lower than those for FGF-1; this is probably due to the increased NaCl concentration present in the assay to limit the nonspecific binding of the protein to the sensor chip.[20] A comparison of the K_D values measured for **2**–**10** bound to FGF-1 shows at most 16-fold variation, with **3** and **7** showing the highest affinity. A comparison of the K_D values for FGF-2 shows a similar variation, at most 12-fold, with **7** again showing the highest affinity. A systematic increase of the length of the alkyl chain separating $NCyc_2$ groups did not result in better binding affinities; this is similar to trends noted in other inhibition studies of these FGF:**1** complexes.[28]

Effect of linker flexibility

The tetracyclitol **11**, in which the alkyl chain is replaced by a *p*-xylyl group, was used to examine the role of linker flexibility in FGF-binding. For example, it can be hypothesized that binding is hindered for ligands with reduced flexibility because positioning sulfate groups for optimal interactions with the binding-site residues is no longer possible. The K_D values measured for **11** (Table 1) do not, however, support such a hypothesis. For both proteins the K_D values for FGF:**11** binding are comparable to those measured for **9** and **10**, which contain the longest and most flexible alkyl linkers. Additionally, they are only two- to fourfold worse than those measured for the tetracyclitols with an alkyl linker of comparable length to **11** (e.g., **5**–**6**). These relatively small changes in K_D indicate that the reduced flexibility of the linker in **11** does not significantly affect its ability to bind to the protein. The observed binding affinities are likely a consequence of the large number of sulfate groups in these ligands, which are able to compensate for any reduced flexibility of the linker and maintain favourable interactions with the FGF.

Effect of linker group

A ligand that incorporates a cyclitol group as part of the linker, **12**, was also considered. This linker incorporates high flexibility and has similar N–N separations to **2** and **6**. The additional sulfates in the linker introduce more negative charges to the

Table 1. Binding affinities of sulfated linked tetracyclitols for FGF-1 and FGF-2. The K_D [nM] values are the weighted averages of two independent measurements and the ΔG(obs) values [kcal mol^{-1}] were calculated according to $\Delta G(\text{obs}) = RT\ln K_D$, where $T = 298$ K.

Ligand	FGF-1		FGF-2	
	K_D	ΔG(obs)	K_D	ΔG(obs)
2	20.7 ± 1.3	−10.48 ± 0.04	1100 ± 7	−8.12 ± 0.04
3	7.2 ± 0.6	−11.10 ± 0.05	248 ± 19	−9.01 ± 0.05
4	25.4 ± 1.6	−10.36 ± 0.04	161 ± 13	−9.26 ± 0.05
5	29.8 ± 1.9	−10.26 ± 0.04	148 ± 4	−9.31 ± 0.03
6	39 ± 3	−10.10 ± 0.05	135 ± 8	−9.37 ± 0.03
7	8 ± 0.7	−11.04 ± 0.05	90 ± 9	−9.61 ± 0.06
8	40.4 ± 2.7	−10.08 ± 0.07	108 ± 7	−9.50 ± 0.04
9	119 ± 7	−9.45 ± 0.03	194 ± 10	−9.15 ± 0.03
10	115 ± 17	−9.46 ± 0.04	448 ± 13	−8.66 ± 0.02
11	187 ± 12	−9.17 ± 0.04	332 ± 12	−8.83 ± 0.02
12	0.40 ± 0.03	−12.81 ± 0.04	85 ± 5	−9.64 ± 0.03

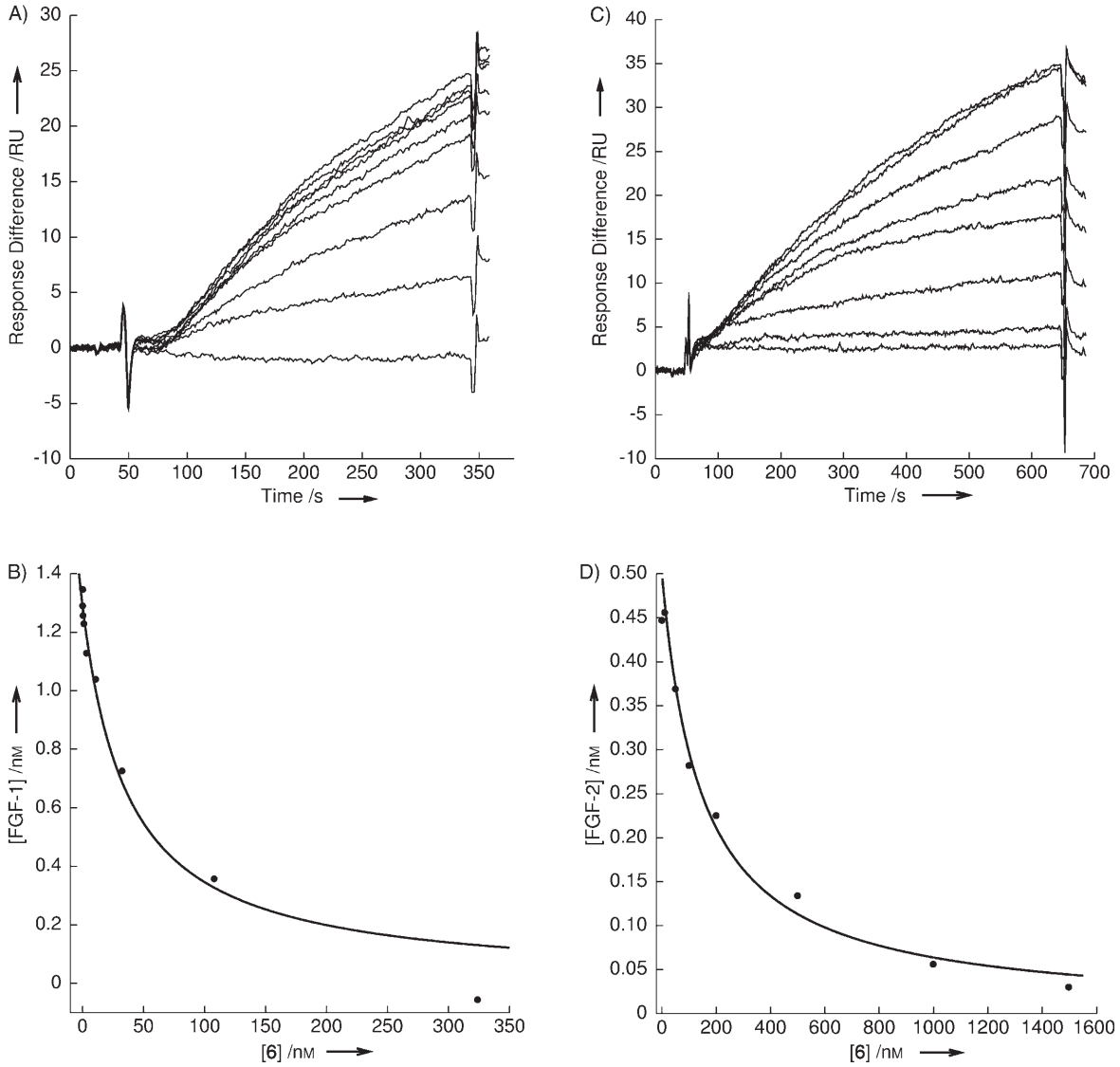


Figure 1. Representative K_D measurements of **6** binding to FGF-1 and FGF-2. A) SPR sensorgrams showing the change in binding response in arbitrary response units (RU) upon injection of 1.29 nM FGF-1 with concentrations of **6** of 0, 0.1, 0.3, 1.0, 3.0, 10.8, 32.4, 107.9 and 323.8 nM (from top to bottom). B) The binding curve showing the concentration of free FGF-1 against the total concentration of **6** and fitting of Equation (3). C) SPR sensorgrams showing the change in binding response (RU) upon injection of 0.5 nM FGF-2 with concentrations of **6** of 0, 0.01, 0.05 nM, 0.1, 0.2, 0.5, 1.0 and 1.5 μM (from top to bottom). D) The binding curve showing the concentration of free FGF-2 against the total concentration of **6** and fitting of Equation (3).

ligand, which might be expected to enhance its interaction with the FGFs. The K_D of **12** binding to FGF-1 was approximately 20-fold lower than for **3** and **7**, the alkyl-linked ligands displaying highest affinities. Thus, a linker comprising an additional sulfated cyclitol affords opportunities for enhanced binding to FGF-1. For FGF-2, the affinity of **12** was similar to that of **7**; this suggests that the presence of an additional sulfated cyclitol neither interferes with nor significantly enhances binding to this protein.

FGF:1 complexes obtained from Monte Carlo Multiple Minima (MCMM) calculations

A Monte Carlo conformational search methodology was applied to the study of FGF:**1** complexes to examine the binding modes of these large, flexible ligands as they are not readily amenable to treatment by conventional molecular docking methods. While the size and flexibility of these ligands preclude an exhaustive search for low-energy complexes with this MCMM method,[29] other studies that used this approach to flexible ligand docking[30–32] indicate that it might nevertheless provide useful information regarding the nature of FGF:**1** binding.

The important result from these MCMM search calculations was the location of plausible binding modes in the HS-binding site of both FGF-1 and FGF-2 for all ligands examined here despite differences in linker size and type. This might be explained by two facts: 1) In both FGFs the binding site is a relatively open, shallow patch on the protein surface. 2) The longer alkyl-chain linker groups confer flexibility on the mole-

cule that allows these ligands to fit within the binding site region. These points are illustrated in Figure 2, which compares the low-energy bound conformations of the cyclitol with the shortest (**2**) and the longest (**10**) linker.

The binding of **2** and **10** to FGF-1 shows that both ligands present sulfate groups to the positively charged residues Lys113, Arg116, Lys118, Arg122 and the neutral residue Asn18. The binding of these ligands to FGF-2 shows that the sulfate groups of both interact with the positively charged residues Lys120, Arg121, Lys126 and Lys130. This result is similar to that obtained from extensive molecular dynamics docking calculations of a heparin-derived tetrasaccharide complexed with FGF-2.[33] In this study, ligand sulfate groups preferentially bound to Lys120, Arg121, Lys126, Lys130 and Lys136. Interestingly, sulfate groups of **2** also interacted with Lys136, as does the cocrystallized heparin-derived tetrasaccharide.[47] In contrast, the longer linker of **10** resulted in interactions with the more distant residue Lys27.

Overlap of cyclitol sulfate positions in the minimum energy FGF:**1** complexes with those sulfates of the cocrystallized ligands—sucrose octasulfate in the case of FGF-1 and a heparin-derived tetrasaccharide in the case of FGF-2—was also noted, in accordance with results of our previous docking calculations for a range of smaller sulfated molecules.[34] This agreement between predicted sulfate locations with those observed crystallographically suggests that the binding modes of the linked cyclitols in the HS-binding site of FGF-1 and FGF-2 predicted here provide some indication of how **1** might bind to these growth factors.

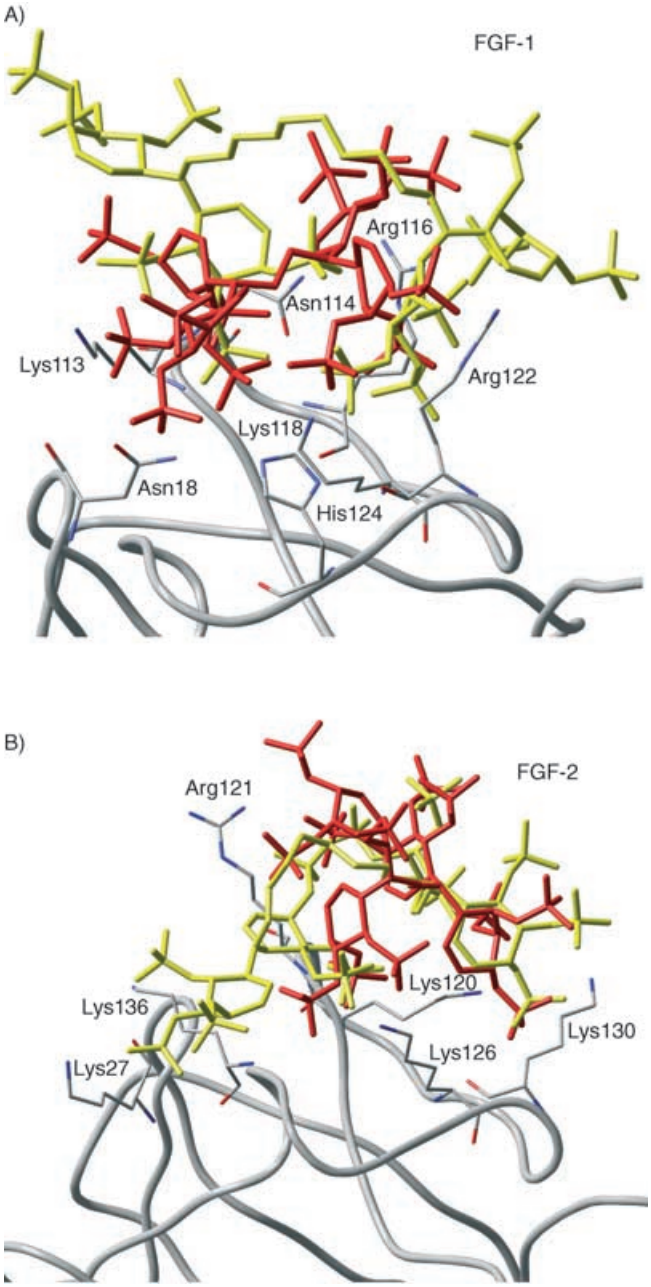


Figure 2. The minimum energy FGF-bound conformations determined by MCMM conformational search calculations for the sulfated tetracyclitol with shortest linker, **2** (red), compared to that with the longest, **10** (yellow). Hydrogen atoms are omitted.

Binding affinities calculated by using LIE(Minim) sampling

The successes of the LIE method are well documented[35–40] and with these in mind it was applied to the FGF:**1** complexes as a means of estimating the FGF-binding affinities for these ligands. The results of calculations performed with the LIE(Minim) sampling methodology are given in Table 2 and plots showing the agreement between observed and predicted binding affinities are shown in Figure 3. The LIE-derived binding affinities are mostly within 1 kcal mol^{-1} of the observed value, although the correlation between ΔG(obs) and ΔG(LIE) is poor, with r^2 values no better than 0.2.

The negative values of α (FGF-2) and β (FGF-1) obtained for the LIE equation also indicates that the LIE(Minim) calculations were not as successful as the small root-mean-square deviation (RMSD) might suggest. Negative coefficients are unphysical and, along with values of β that deviate significantly from ideal, indicate problems with the calculation of the electrostatic contributions to ΔG(LIE).[41] Although the physicality of the LIE equation could be ignored and treated simply as a structure–activity relationship, the dominant γ coefficient along

Table 2. Results of the LIE calculations. α, β, and γ are the coefficients used in Equation (7) to obtain the best fit of ΔG(obs) to ΔG(LIE). RMSD denotes the root mean square deviation of this best fit.

	FGF-1				FGF-2			
	α	β	γ	RMSD	α	β	γ	RMSD
Minim	0.0086	−0.0098	4.82	1.01	−0.0503	0.0052	2.56	0.73
HMC	−0.0270	−0.0082	4.97	1.08	−0.0409	0.0044	2.35	0.76
HMC+Na^+	0.1239	0.0078	2.13	0.96	0.0825	0.0063	1.01	0.82

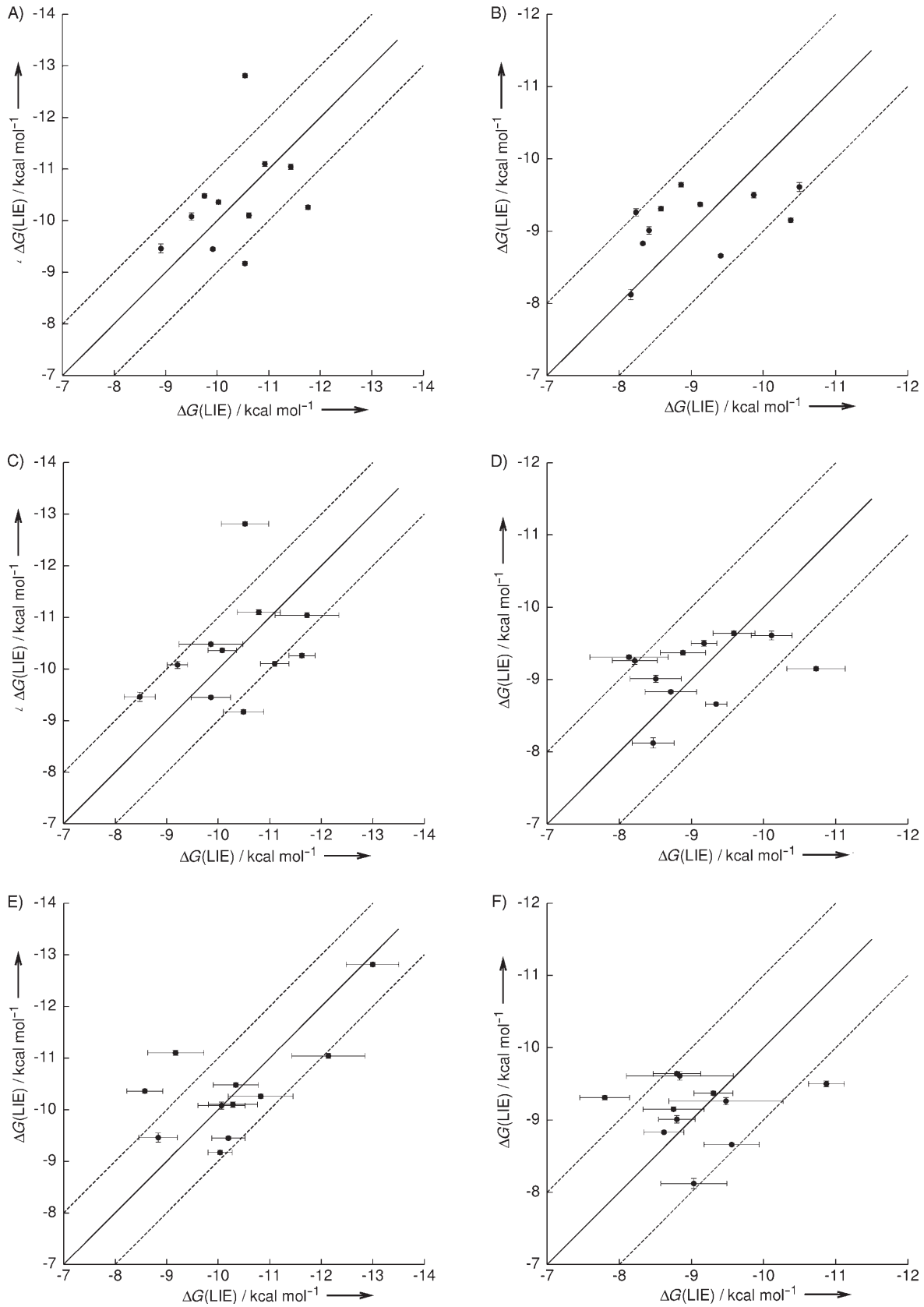


Figure 3. Plots of ΔG(obs) versus ΔG(LIE) obtained from various LIE calculations. In each plot the full line indicates ideal agreement between experiment and theory while the dashed lines bound the region within 1 kcal mol^{-1} of ideal. A) FGF-1, LIE(Minim); B) FGF-2, LIE(Minim); C) FGF-1, LIE(HMC); D) FGF-2, LIE(HMC); E) FGF-1, LIE(HMC+Na^+); F) FGF-2, LIE(HMC+Na^+).

with the small and possibly negative α and β terms indicated that further exploration of the LIE method was warranted for these FGF:**1** complexes.

Binding affinities calculated by using the LIE(HMC) method

The results of the LIE(HMC) calculations mirror those obtained with the LIE(Minim) method. The LIE(HMC) coefficients (Table 2) are similar and the correlation coefficients for both FGF:**1** systems are also low, approximately 0.1. This is clearly shown in Figure 3, which also shows error estimates for the calculation of ΔG(LIE) when HMC sampling was used. Consideration of these error bars, which are similar in magnitude to estimates derived in a recent LIE study,[42] place the majority of ligands within 1 kcal mol^{-1} deviation from ideal values, although the unphysical LIE equation persists.

The similarity between the LIE(Minim) and LIE(HMC) results is in accordance with an earlier study,[40] which showed that the extra effort of HMC sampling did not result in better agreement with experimental data as compared to the values obtained by collecting the LIE energy components after energy minimization of the protein–ligand complex. The reasons for the unphysical LIE equation, therefore, cannot be attributed to the method used to obtain the component energies and, as stated above, is likely due to overestimation of the electrostatic component of the binding affinity.

To test this hypothesis each FGF:**1** complex was neutralized by the addition of sufficient Na^+ counter ions followed by HMC sampling to obtain the LIE component energies. The coefficients obtained from these LIE(HMC+Na^+) calculations (Table 2) are all positive suggesting that the addition of the cations helped reduce the electrostatic component of ΔG(LIE) to a more reasonable estimate. The plots in Figure 3 show slightly better correlation in the case of FGF-1 (Figure 3E), while the FGF-2 results (Figure 3F) show similar scatter to those already described. Nevertheless, the trend towards a more physical LIE equation for both FGF:**1** systems upon neutralization of the complex through the addition of Na^+ counterions is encouraging.

Effect of protein concentration on binding of 6 and 10 to the FGFs

In light of the relatively long and flexible nature of these sulfated tetracyclitol ligands, it might be hypothesized that the two ends of a sulfated tetracyclitol ligand bind independently to different FGF molecules. The resultant complex would have a 2:1 stoichiometry with the ligand effectively cross-linking two protein molecules. Such cross-linking of growth factors resulting in growth-factor aggregation has been attributed to suramin, which also contains two sulfated moieties.[43] Because the solution-affinity assay employed here is specific for the HS-binding site such a cross-linking binding mechanism, which is dependent on protein concentration (see Equation (6) in the Experimental Section), should be detectable.

To test this hypothesis, the binding affinity measurements were repeated for **6** and **10** by using a 20–60-fold higher concentration of FGF-1 or FGF-2. No significant changes in K_D values were observed (Table 3); this indicates a 1:1 stoichiometry for these ligands in complex with FGF-1 and FGF-2. Thus, cross-linking of two FGF molecules by the sulfated tetracyclitols is not apparent. The observed binding affinities appear to originate from the interaction of the tetracyclitol with residues in the HS-binding site of a single FGF.

Table 3. Comparison of affinities of **6** and **10** binding to FGF-1 and FGF-2, measured at low and at 20–60-fold higher protein concentrations. The average K_D of two or more independent measurements is shown for the low protein concentration measurements, whereas the K_D at high protein concentration was measured once only. Protein concentrations and K_D values are given in nM.

FGF	[FGF-1]		[FGF-2]	
concentration	1.29	25.8	0.50	30.0
K_D (**6**)	75.4 ± 9.6	136 ± 5	139 ± 10	384 ± 33
K_D (**10**)	141 ± 33	89.0 ± 4.0	469 ± 44	659 ± 59

Conclusion

This study has focused on a series of linked sulfated tetracyclitols binding to FGF-1 and FGF-2. By using a solution-affinity assay previously devised by us[20] K_D values for the interaction of these ligands with both FGFs were determined. All ligands bind to FGF-1 and FGF-2 with nM or even pM affinities, similar to those previously measured for low-molecular-weight heparin fragments.[20] In addition, little variation in binding affinity is observed upon increasing the length of the flexible alkyl chain or reducing its flexibility through the introduction of a *p*-xylyl linker.

A search for low-energy conformations of the FGF-bound tetracyclitol ligands showed how the tetracyclitols might be accommodated in the HS-binding sites of FGF-1 or FGF-2 irrespective of the type or length of functional group connecting the $NCyc_2$ ends. Furthermore, these ligands bind in a manner that places sulfate groups in regions of the FGF-binding sites occupied by those of the cocrystallized ligands.

The estimation of binding affinities by using the LIE method met with modest success. Initial calculations produced agreement with experimental values to within an RMSD of 1 kcal mol^{-1} at the expense of unreasonable α, β and γ LIE coefficients. Although the RMSD agreement was not significantly improved, LIE coefficients with improved physical meaning were obtained from calculations that included enough Na^+ ions to give FGF:**1** complexes with overall neutral charge.

The possibility that these potentially bifunctional ligands might bind to two independent FGFs was explored by performing the solution-affinity assay under conditions of greatly increased protein concentrations. Under such conditions, the formation of higher-order FGF:**1** complexes would result in significantly different K_D values, and as these were not observed, such cross-linking appears unlikely.

The involvement of the FGFs in tumour angiogenesis makes them an attractive target for cancer therapy, and the design of ligands that compete for HS-binding is one approach to inhib-

iting cancer cell proliferation activity. Our experimental studies confirm the binding of these ligands in the HS-binding site, while our computational studies demonstrate possible binding modes for these ligands, and LIE calculations help rationalize the experimental binding affinities. From this combined study, we conclude that the binding affinities of these tetracyclitol molecules do not depend on linker length or flexibility, although the presence of an additional sulfated cyclitol in the linker is favourable, particularly for binding to FGF-1. These results suggest how future design of HS mimetics can proceed by using a combination of theoretical and experimental studies.

Experimental Section

K_D measurement

Materials: Human FGF-1 (140 residues, *N*-terminally truncated form) and human FGF-2 (146 residues, *N*-terminally truncated form) were purchased from R&D Systems, Inc. and supplied as protein (1 μg) dissolved in bovine serum albumin (BSA, 50 μg). Surface plasmon resonance (SPR) measurements were performed on a BIAcore 3000 (BIAcore, Uppsala, Sweden) operated by the BIAcore control software (version 3.1). HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.0 mM EDTA, 0.005% *v/v* polysorbate 20), CM4, CM5 and streptavidin-coated sensor chips were purchased from BIAcore.

Ligand-affinity measurements: Binding affinities of the sulfated tetracyclitol ligands (**2**–**12**) for FGF-1 and FGF-2 were measured by using a solution affinity assay described previously.[20] This assay uses immobilized heparin to distinguish between free and bound growth factor in an equilibrated solution of growth factor and a ligand. Different heparin-coated sensor chips were prepared, either by immobilization of biotinylated BSA-heparin on a streptavidin-coated sensor chip[20] or by aldehyde coupling via the reducing end of heparin to CM4 or CM5 sensor chips.[44] These chips were used interchangeably for all measurements because earlier studies showed that K_D value determination was independent of the chip type used for the assay.[44]

Upon injection of the equilibrated FGF–ligand solution the binding of the free growth factor to the immobilized heparin was detected as an increase in the SPR response. From this, the free growth-factor concentration can be determined. Results from negative control flowcells (albumin–biotin immobilized on streptavidin sensor chips or untreated CM4 and CM5 sensor chips) were subtracted from the heparin-immobilized cells. Data were normalized to zero relative response units at the beginning of the spectra. The dissociation constant, K_D, was calculated from the decrease in the free growth factor concentration as a function of the ligand concentration.

For each K_D measurement solutions of FGF-1 (1.3 nM) or FGF-2 (0.5 nM) and varying concentrations of the ligand were prepared in buffer. Ligand binding to FGF-1 was measured in HBS-EP buffer, while binding to FGF-2 was measured in HBS-EP buffer with an increased concentration of NaCl (0.3 M).[20] Prior to injection, samples were maintained at 4 °C to maximize protein stability, however, the surface binding experiments were performed at 25 °C. For each assay the equilibrated FGF–ligand solution (50–200 μL) was injected (5–40 μL min^{-1}) and the relative binding response was measured. The variation in flowrate does not affect the final K_D values since mass transport conditions were observed for the entire range of flowrates. The sensor chip surface was regenerated by injection of NaCl (40 μL, 4 M, 40 μL min^{-1}) followed by injection of buffer (40 μL, 40 μL min^{-1}). All K_D values were measured in duplicate unless otherwise indicated.

A stoichiometry of 1:1 was assumed for the protein–ligand complex formed in solution prior to injection, given by Equation (1),

$$P + L \rightleftharpoons PL \quad (1)$$

where P corresponds to the FGF, L is the ligand **1** (Scheme 1), and PL is the FGF:**1** complex. The Equation (2) for the equilibrium constant is

$$K_D = \frac{[P][L]}{[PL]} \quad (2)$$

and Equation (3) relating K_D to free protein concentration can be expressed as:

$$[P] = [P]_{total} - \frac{K_D + [L]_{total} + [P]_{total}}{2} + \sqrt{\frac{(K_D + [L]_{total} + [P]_{total})^2}{4} - [L]_{total}[P]_{total}} \quad (3)$$

where $[P]_{total}$ and $[L]_{total}$ represent the total concentrations of protein and ligand, respectively, in the injected solution.[20]

Under conditions of mass transport, standard curves relating the relative binding response to the injected protein concentration are linear.[45] The relative binding response for each injection can, therefore, be converted to free protein concentration by using Equation (4),

$$[P] = \frac{r}{r_m}[P]_{total} \quad (4)$$

where r is the relative binding response and r_m is the maximal binding response in the absence of ligand. A plot of [P] versus $[L]_{total}$ and fitting of Equation (3) enables the determination of the K_D.

The possibility of higher stoichiometries in the FGF:**1** complex was also investigated by postulating an equilibrium of the type given in Equation (5),

$$nP + mL \rightleftharpoons P_nL_m \quad (5)$$

where P_nL_m represents a complex in which n protein molecules are bound to m ligand molecules. In this case, the resultant or apparent K_D is given by Equation (6).

$$K_D^{app} = \frac{[P]^n[L]^m}{[P_nL_m]} \quad (6)$$

The presence of higher order complexes might, therefore, become evident if K_D^{app} is significantly different from K_D when the concentration of FGF is altered. In our experiments, the protein concentration was increased 20–60-fold to provide a sufficiently large change in K_D^{app} if FGF:**1** complexes of stoichiometries other than 1:1 were present in the equilibrated solutions.

Molecular modelling: The X-ray structures with pdb accession codes 1AFC[46] and 1BFB[47] were used to model FGF-1 and FGF-2, respectively. Both protein structures were prepared for calculations by using a previously described method[34] that involved protonation to yield an overall octapositive charge to balance the negative charge of the cocrystallized ligand, followed by restrained energy

minimization to orient side-chain hydroxyl groups and relieve steric clashes.

Monte Carlo multiple minima (MCMM) calculations: Low-energy conformations of the sulfated linked tetracyclitol ligands **1** complexed with FGF-1 and FGF-2 were explored by using the MCMM method[48] in conjunction with the OPLS-AA forcefield,[49] as implemented in the MacroModel program (version 8.1).[50] Partial charges for all atoms were assigned by using this forcefield and solvation in water was accounted for by the GB/SA continuum solvation model.[51]

MCMM calculations to locate the lowest energy binding conformations of each ligand were performed in several stages. After construction and energy minimization, the sulfated tetracyclitol ligand (fully ionized and with no Na^+ counter ions) was placed manually in the binding site by using the cocrystallized ligand (sucrose octasulfate (SOS) for FGF-1 and a heparin-derived tetrasaccharide for FGF-2) or a previously generated ligand conformation as a guide. The geometry of each preliminary FGF:**1** complex was subsequently relaxed until the gradient was less than 0.01 kcal mol^{-1} $Å^{-1}$ by allowing full freedom of movement to the tetracyclitol ligand, but not the protein.

The initial FGF:**1** complexes were used to start a sequence of MCMM calculations during which the conformational flexibility of only the ligand was explored. Furthermore, the relative orientations of the ligand sulfate groups on the cyclitol rings were not allowed to change during conformational searching. The stereochemistries of the sulfate groups in **1** were therefore maintained throughout all MCMM calculations. Residues within 15 Å of the tetracyclitol ligand were included in the evaluation of the energy of the FGF:**1** complex during the MCMM calculations.

Several stages of MCMM calculations were performed to find plausible low-energy FGF:**1** complexes. Each stage consisted of 1000 Monte Carlo steps with the lowest energy conformer obtained from each used as the seed for the next 1000-step MCMM calculation. The sequence was stopped when the lowest energy conformer differed in energy from the input conformer by less than 1 kcal mol^{-1}. In all cases at least three rounds (i.e., 3 by 1000 steps) of Monte Carlo calculations were performed, though in some cases as many as seven were required.

LIE binding-affinity calculations: The theoretical binding affinities of the lowest energy FGF:**1** complexes obtained from the MCMM calculations were estimated with the LIE method,[39] as implemented in *Liaison* (v3.0).[52,53] The OPLS-2001 forcefield[49] and solvent effects incorporated via the surface generalized Born (SGB) continuum solvation model[54] were used in these calculations. The LIE equation is defined by Equation (7),

$$\Delta G(\mathrm{LIE}) = \alpha\langle\Delta U_{\mathrm{vdw}}\rangle + \beta\langle\Delta U_{\mathrm{elec}}\rangle + \gamma\langle\Delta U_{\mathrm{cav}}\rangle \quad (7)$$

where ΔU_{vdw}, ΔU_{elec} and ΔU_{cav} are the differences between the bound and free averages of the van der Waals (vdw), electrostatic (elec) and cavity (cav) energies. The coefficients α, β and γ are determined by fitting the various ΔU quantities to the ΔG(obs) values by using the single value decomposition method implemented within *Liaison*.

During the course of LIE calculations the FGF:**1** complex was divided into three regions:

Active: the ligand and, where relevant, Na^+ counterions and amino acids within 12 Å of the cocrystallized ligand. Atoms in these regions are allowed to move freely during both geometry minimization and LIE simulation.

Buffered: protein residues between 12 Å and 18 Å of the cocrystallized ligand. The atoms in this region were constrained by a force constraint of 25 kcal $Å^{-2}$ mol^{-1}.

Frozen: those protein residues greater than 18 Å from the cocrystallized ligand were not allowed to move

The definition of these regions relative to the location of the cocrystallized ligand ensured that the same residues defined each region in all calculations.

Prior to energy sampling each complex was relaxed by conjugate gradient minimization with the complex divided into the different regions described above. Complexes were considered converged when their change in energy was less than 10^{-5} kcal mol^{-1} and root mean square forces were less than 0.05 kcal mol^{-1} $Å^{-1}$ (protein–ligand complex) or 0.01 kcal mol^{-1} $Å^{-1}$ (free ligand). A residue-based cutoff of 25 Å distance was used to define the maximum distance for considering pairwise interactions.

Two sampling methods were employed to calculate the different ΔU terms in Equation (7). The first, denoted LIE(Minim), involved collection of LIE energies upon completion of the energy minimization step. The second, denoted LIE(HMC), involved HMC calculations for a duration of 50 ps: 20 ps of heating and 30 ps of simulation. These HMC calculations were performed at a target temperature of 300 K using 0.002 ps time steps and 5 molecular dynamics steps per HMC cycle. Energies averaged over each 0.01 ps were monitored during the simulation to assess convergence. The errors in each energy component derived from this averaging were propagated to yield error bars in the final calculated binding affinities ΔG(LIE). Errors were also evaluated as the difference in energies at the final and halfway points of the simulation in the manner described by Almlöf et al.[42] As this procedure yielded similar, though slightly smaller error values, only the larger error estimates are presented.

LIE calculations were also performed on the FGF:cyclitol complexes neutralized by the addition of Na^+ counterions (4 for **2**–**11**, 7 for **12**). These counterions were placed randomly near the ligand and were included in the active region during the minimization and HMC sampling steps of the LIE calculation. To achieve acceptable convergence the equilibration and simulation times for the free ligand were doubled, whereas simulation times for all complexes, with the exception of FGF-1–**11**, were the same as for the simulations without the inclusion of Na^+.

Plots of the averaged LIE terms versus the HMC step are available for all calculations as Supporting Information.

Acknowledgements

We are grateful to Prof. Martin Banwell and Dr. Ligong Liu for providing us with the sulfated linked tetracyclitol ligands. We also thank Dr. M. Shelley and Dr. D. Bernhardt for technical assistance with the molecular modelling; Dr. Vito Ferro for critical reviewing of the manuscript; and our Drug Design Group colleagues for their helpful discussions and suggestions during the course of this work. This project was funded in part by an Aus-Industry Start grant.

Keywords: angiogenesis · drug design · fibroblast growth factor · molecular modeling · surface plasmon resonance

[1] J. Folkman, M. Klagsbrun, *Science* **1987**, *235*, 442 – 447.
[2] P. Carmeliet, *Nat. Med.* **2003**, *9*, 653 – 660.
[3] D. M. Ornitz, A. Yayon, J. G. Flanagan, C. M. Svahn, E. Levi, P. Leder, *Mol. Cell. Biol.* **1992**, *12*, 240 – 247.
[4] T. Spivak-Kroizman, M. A. Lemmon, I. Dikic, J. E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumley, J. Schlessinger, I. Lax, *Cell* **1994**, *79*, 1015 – 1024.
[5] J. Zhang, G. Rivers, Y. Zhu, A. Jacobson, J. Peyers, G. Grundstrom, P. Burch, S. Hussein, A. Marolewski, W. Herlihy, J. Rusche, *Bioorg. Med. Chem.* **2001**, *9*, 825 – 836.
[6] L. Liu, C. Ping Li, S. Cochran, V. Ferro, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2221 – 2226.
[7] C. Tabeur, J.-M. Mallet, F. Bono, J.-M. Herbert, M. Petitou, P. Sinaÿ, *Bioorg. Med. Chem.* **1999**, *7*, 2003 – 2012.
[8] M. Presta, D. Leali, H. Stabile, R. Ronca, M. Camozzi, L. Coco, E. Moroni, S. Liekens, M. Rusnati, *Curr. Pharm. Des.* **2003**, *9*, 553 – 566.
[9] A. Bentolila, I. Vlodavsky, R. Ishai-Michaeli, O. Kovalchuk, C. Haloun, A. J. Domb, *J. Med. Chem.* **2000**, *43*, 2591 – 2600.
[10] S. Liekens, D. Leali, J. Neyts, R. Esnouf, M. Rusnati, P. Dell'Era, P. C. Maudgal, E. De Clercq, M. Presta, *Mol. Pharmacol.* **1999**, *56*, 204 – 213.
[11] M. Benezra, R. Ishai-Michaeli, S. A. Ben-Sasson, I. Vlodavsky, *J. Cell. Physiol.* **2002**, *192*, 276 – 285.
[12] D. Jellinek, C. K. Lynott, D. B. Rifkin, N. Janjic, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11 227 – 11 231.
[13] P. V. Murphy, N. Pitt, A. O'Brien, P. M. Enright, A. Dunne, S. J. Wilson, R. M. Duane, K. M. O'Boyle, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3287 – 3290.
[14] A. Wellstein, G. Zugmaier, J. A. Califano, F. Kern, S. Paik, M. E. Lippman, *J. Natl Cancer Inst.* **1991**, *83*, 716 – 720.
[15] C. Foxall, Z. Wei, M. E. Schaefer, M. Casabonne, P. Fugedi, C. Peto, J. J. Castellot, B. K. Brandley, *J. Cell. Physiol.* **1996**, *168*, 657 – 667.
[16] B. Casu, M. Guerrini, A. Naggi, M. Perez, G. Torri, D. Ribatti, P. Carminati, G. Giannini, S. Penco, C. Pisano, M. Belleri, M. Rusnati, M. Presta, *Biochemistry* **2002**, *41*, 10 519 – 10 528.
[17] M. A. Rosenthal, D. Rischin, G. McArthur, K. Ribbons, B. Chong, J. Fareed, G. Toner, M. D. Green, R. L. Basser, *Ann. Oncol.* **2002**, *13*, 770 – 776.
[18] V. Ferro, R. Don, *Australas. Biotechnol.* **2003**, *13*, 38 – 39.
[19] D. J. Francis, C. R. Parish, M. McGarry, F. S. Santiago, H. C. Lowe, K. J. Brown, J. A. Bingley, I. P. Hayward, W. B. Cowden, J. H. Campbell, G. R. Campbell, C. N. Chesterman, L. M. Khachigian, *Circ. Res.* **2003**, *92*, 70e – 77e.
[20] S. Cochran, C. Li, J. K. Fairweather, W. C. Kett, D. R. Coombe, V. Ferro, *J. Med. Chem.* **2003**, *46*, 4601 – 4608.
[21] C. R. Parish, C. Freeman, K. J. Brown, D. J. Francis, W. B. Cowden, *Cancer Res.* **1999**, *59*, 3433 – 3441.
[22] E. J. Small, M. Meyer, M. E. Marshall, L. M. Reyno, F. J. Meyers, R. B. Natale, P. F. Lenehan, L. Chen, W. J. Slichenmyer, M. Eisenberger, *J. Clin. Oncol.* **2000**, *18*, 1440 – 1450.
[23] F. Manetti, F. Corelli, M. Botta, *Curr. Pharm. Des.* **2000**, *6*, 1897 – 1924.
[24] G. P. Schneider, R. Salcedo, H. F. Dong, H. K. Kleinman, J. J. Oppenheim, O. M. Howard, *Clin. Cancer Res.* **2002**, *8*, 3955 – 3960.
[25] R. M. Lozano, M. Á. Jiménez, J. Santoro, M. Rico, G. Giménez-Gallego, *J. Mol. Biol.* **1998**, *281*, 899 – 915.
[26] C. Fernández-Tornero, R. M. Lozano, M. Redondo-Horcajo, A. M. Gómez, J. C. López, E. Quesada, C. Uriel, S. Valverde, P. Cuevas, A. Romero, G. Giménez-Gallego, *J. Biol. Chem.* **2003**, *278*, 21 774 – 21 781.
[27] M. G. Banwell, L. Liu, C. R. Parish, C. G. Freeman, *PCT Int. Appl. WO 03/004454*; *Chem. Abstr.* **2003**, *138*, 90 018.
[28] C. Freeman, L. Liu, M. G. Banwell, K. J. Brown, A. Bezos, V. Ferro, C. R. Parish, *J. Biol. Chem.* **2005**, *280*, 8842 – 8849.
[29] G. Keserü, I. Kolossváry, *Molecular Mechanics and Conformational Analyses in Drug Design*, Blackwell Science, Oxford, **1999**.
[30] B. Botta, G. Zappia, A. Tafi, M. Botta, F. Manetti, E. Cernia, G. Milana, C. Palocci, S. Soro, G. D. Monache, *J. Mol. Catal. B* **2002**, *16*, 241 – 247.
[31] M. Botta, F. Corelli, F. Manetti, A. Tafi, *Farmaco* **2002**, *57*, 153 – 165.
[32] F. Manna, F. Chimenti, A. Bolasco, D. Secci, B. Bizzarri, O. Befani, P. Turini, B. Mondoví, S. Alcaro, A. Tafi, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3629 – 3633.
[33] E. Krieger, E. Geretti, B. Brandner, B. Goger, T. N. Wells, A. J. Kungl, *Proteins* **2004**, *54*, 768 – 775.
[34] I. Bytheway, S. Cochran, *J. Med. Chem.* **2004**, *47*, 1683 – 1693.
[35] I. D. Wall, A. R. Leach, D. W. Salt, M. G. Ford, J. W. Essex, *J. Med. Chem.* **1999**, *42*, 5142 – 5152.
[36] D. K. Jones-Hertzog, W. L. Jorgensen, *J. Med. Chem.* **1997**, *40*, 1539 – 1549.
[37] A. C. Pierce, W. L. Jorgensen, *J. Med. Chem.* **2001**, *44*, 1043 – 1050.
[38] R. C. Rizzo, J. Tirado-Rives, W. L. Jorgensen, *J. Med. Chem.* **2001**, *44*, 145 – 154.
[39] J. Åqvist, V. B. Luzhkov, B. O. Brandsdal, *Acc. Chem. Res.* **2002**, *35*, 358 – 365.
[40] B. A. Tounge, C. H. Reynolds, *J. Med. Chem.* **2003**, *46*, 2074 – 2082.
[41] J. Åqvist, J. Marelius in *Free Energy Calculations in Rational Drug Design* (Eds.: M. R. Reddy, M. D. Erion), Kluwer Academic/Plenum, New York, **2001**, pp. 171 – 194.
[42] M. Almlöf, B. O. Brandsdal, J. Åqvist, *J. Comput. Chem.* **2004**, *25*, 1242 – 1254.
[43] C. R. Middaugh, H. Mach, C. J. Burke, D. B. Volkin, J. M. Dabora, P. K. Tsai, M. W. Bruner, J. A. Ryan, K. E. Marfia, *Biochemistry* **1992**, *31*, 9016 – 9024.
[44] C. Li, S. Cochran, V. Ferro, unpublished results.
[45] R. Karlsson, H. Roos, L. Fägerstam, B. Persson, *Methods Companion Methods Enzymol.* **1994**, *6*, 99 – 110.
[46] X. Zhu, B. T. Hsu, D. C. Rees, *Structure* **1993**, *1*, 27 – 34.
[47] S. Faham, R. E. Hileman, J. R. Fromm, R. J. Linhardt, D. C. Rees, *Science* **1996**, *271*, 1116 – 1120.
[48] G. Chang, W. C. Guida, W. C. Still, *J. Am. Chem. Soc.* **1989**, *111*, 4379 – 4386.
[49] W. L. Jorgensen, D. S. Maxwell, J. Tirado-Rives, *J. Am. Chem. Soc.* **1996**, *118*, 11 225 – 11 236.
[50] F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson, W. C. Still, *J. Comput. Chem.* **1990**, *11*, 440 – 467.
[51] W. C. Still, A. Tempczyk, R. C. Hawley, T. Hendrickson, *J. Am. Chem. Soc.* **1990**, *112*, 6127 – 6129.
[52] *FirstDiscovery Technical Notes*; Schrödinger, Portland, **2003**.
[53] R. Zhou, R. A. Friesner, A. Ghosh, R. C. Rizzo, W. L. Jorgensen, R. M. Levy, *J. Phys. Chem. B* **2001**, *105*, 10 388 – 10 397.
[54] A. Ghosh, C. S. Rapp, R. A. Friesner, *J. Phys. Chem. B* **1998**, *102*, 10 983 – 10 990.

Received: March 3, 2005

