Characterization of the Allosteric Inhibition of a Protein–Protein Interaction by Mass Spectrometry

Walter Davidson, Jerry L. Hopkins, Deborah D. Jeanfavre, Kathleen Last Barney, Terence A. Kelly, and Christine A. Grygon
Research and Development Center, Boehringer Ingelheim Pharmaceuticals, Ridgefield, Connecticut, USA

The allosteric inhibition of the lymphocyte function associated antigen-1/intercellullar adhesion molecule (LFA-1/ICAM-1) interaction, by a class of small molecules, is characterized by a battery of mass spectrometric techniques. Binding of hydantoins to the I domain of LFA-1 is observed by size exclusion chromatography/mass spectrometry (SEC/MS) and by direct electrospray ionization mass spectrometry (ESI/MS). A photoactive hydantoin analog specifically labels an amino acid residue of LFA-1 I domain. Competition with this photoaffinity labeling by a panel of inhibitors is correlated with their K_d's for inhibition of the LFA-1/ICAM interaction. Alterations to the tertiary structure of LFA-1 I domain, upon compound binding, are inferred from perturbation in the ESI mass spectrum of the polypeptide's charge state distribution and by an altered level of nonspecific multimer formation. The results demonstrate specific, stoichiometric, reversible binding of the hydantoins to LFA-1. They further show correlation of this binding with activity and indicate alterations in the polypeptide's tertiary structure, on hydantoin binding, consistent with the proposed mechanism for inhibition of the protein–protein interaction. (J Am Soc Mass Spectrom 2003, 14, 8–13) © 2003 American Society for Mass Spectrometry

The study of inhibition of a protein-protein interaction by a small molecule is a particularly challenging task. When putative inhibitors are identified via high throughput screening (HTS) technologies it is essential to characterize the mechanism of their inhibition so that inhibitors operating via various undesirable modes of action (for development of a therapeutic drug), such as nonspecific binding, irreversible binding, etc. can be efficiently eliminated. These concerns prompted the studies of the hydantoins' mode of action described herein. General and efficient means are required to eliminate all but the most promising hits from a HTS campaign (the BI screening library contains several hundred thousand small molecules). It is also useful to obtain an indication that binding will be sufficient to produce the desired inhibitory effect.

LFA-1 is a member of the β2-integrin family of proteins and plays a pivotal role in directing proinflammatory cells into sites of inflammation [1–4]. Antagonists of this protein have therapeutic potential for several inflammatory and autoimmune diseases. The hydantoin, BIRT377 (CMPD 12), is a potent antagonist of LFA-1 [5]. The compound exerts its influence by binding to a region on LFA-1 (I- or "inserted"-domain) and preventing a conformational change necessary for LFA-1 to bind to its ligands, the intercellular adhesion molecules (ICAM's) [6].

We have reported the elucidation of the ligand-binding site of a novel class of antagonists of LFA-1. The binding site was identified in the absence of cocrystallization studies by docking the inhibitor into a known crystal structure of the apo-protein. Photoaffinity labeling (PA) was combined with enzymatic digestion and mass spectrometric analyses to identify the amino acid residue where photo attachment occurred [7]. This information was a critical component of the successful modeling effort.

Several mass spectrometric techniques are available for the study of the mode of action of small molecule leads identified by high capacity screens. In general, the MS based technologies have the advantage that only small amounts of protein reagent are required. The direct observation of the small molecule binding to the complex or a portion of the complex is an important tool. This is frequently accomplished by ESI/MS conducted under nondenaturing conditions [8–14]. The charge distribution of the electrospray spectrum contains important information related to the preservation of intramolecular bonds and tertiary structure [15, 16]. ESI/MS spectra have been employed to characterize specific, multimeric protein structures [17–21].

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Address reprint requests to Dr. W. Davidson, Research and Development Center, Boehringer Ingelheim Pharmaceuticals, 900 Ridgebury Road P.O. Box 368, Ridgefield, CT 06877-0368, USA. E-mail: wdavidso@rdg.boehringer-ingelheim.com

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Two methods in which mass spectrometry is employed for detection in combination with other procedures were employed in this work. The use of SEC to separate the protein/small molecule complex from unbound small molecules is combined with LC/MS detection of the small molecules that are subsequently released from the denatured complex [20–23]. This technique was employed to verify binding of the small molecule to LFA-1 I domain. Photoaffinity labeling allows a specific, noncovalent interaction to be "frozen" by creation of a covalent bond. This is accomplished by incubation of a photoreactive, benzophenone analog of the small molecule with the polypeptide and subsequent irradiation [24, 25]. Tryptic digestion and LC/MS analysis then determined the location and extent of labeling. [7]. In this report, the PA procedure is expanded to provide a semiquantitative monitoring of competitive binding. This allowed a correlation with the results obtained from methods which directly measured the inhibition of the protein–protein interaction between LFA-1 and ICAM.

Experimental

SEC/MS

The I domain of LFA-1 (rCD11a I domain, construct KLB14.2.1) was prepared as previously reported [7] at a concentration of 32 μM in 10 mM TRIS, at pH 8 with 5 mM $MnCl_2$ and 5 mM β-mercaptoethanol. Stock solutions of CMP1, CMPD 2, and CMPD 4 were prepared as 75 μM solutions in DMSO. 1 ul aliquots of the three small molecule inhibitors were combined with a 20 μl aliquot of the I domain of LFA-1 or a corresponding buffer blank. 10 μl injections, of these solutions, were made and directed to the SEC column for trapping (from 24–48 s at 4°C) and subsequent RPHPLC/MS or directly to RPHPLC/MS for determination of response factors and retention times. The apparatus and switching valve arrangement is essentially similar to that previously reported [22]. Competitive photoaffinity was conducted as previously described [7].

Direct ESI

Recombinant LFA-1 I domain was prepared as previously described [7]. The preparation was dialyzed overnight versus water to produce an aqueous solution 40 μM in I domain. 20 μl aliquots of this solution were diluted with 2 μl of 0.1 M NH_4OAc at pH 7. Incubation with aqueous solutions of the various small molecules was performed by adding ca. 4 μl of each in aqueous solution, containing 2% DMSO. The final solutions were thus ca. 30.7 μM in I domain, 0.008M NH_4OAc, 0.3% DMSO. Putative inhibitors are present at about 1.1 times molar excess to I domain unless otherwise stated. The samples were analyzed by direct infusion ESI/MS into an AutoSpec OATOF mass spectrometer (Micromass, Manchester, UK) at 5 μl/min. The instrument was scanned from *m/z* 5000–1000 at 8 s/decade. Scans were averaged in Profile mode. The skimmer and cone voltages gave optimum preservation of the noncovalent attachment at 12 and 15V. The source temperature was 77 °C. The ratio of peak heights for the denatured monomer/native monomer was calculated as the average height of the +15 to +17 charge states/height of +8 charge state. The ratio of native multimer/native monomer was calculated as the summed area of the +9 and +8 charge states divided by the summed area of the multimer peaks above $m/z = 3200$. The response from $m/z = 3000$–3200 may contain both dimer and monomer response and was therefore not used.

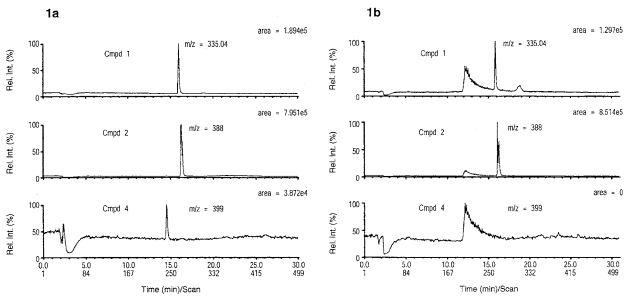

Figure 1. Mass chromatograms for SEC/RPHPLCMS of three hydantoins incubated with LFA-1 I domain. (**a**) is the control to measure retention times and MS response. (**b**) shows the experimental response after incubation, SEC, trapping, and RPHPLCMS. Binding is thus observed for CMPD 1 and CMPD 2 and not observed for CMPD 4. The broad peak observed at 12 min in (**b**) is due to dissociated I domain.

Results and Discussion

Observation of Small Molecule Binding by SEC/MS

The binding of hydantoin inhibitors to LFA-1 I domain was demonstrated by SEC combined with RP HPLC/MS. In this method, small molecules are first incubated with polypeptide target. Size exclusion chromatography is then employed to rapidly separate the polypeptide target, along with any small molecules bound to the target, from any unbound small molecules. The early eluting portion of the chromatogram containing the target and bound molecules is trapped. This trapped fraction is subjected to RP HPLC/MS. This serves to denature the target, thus releasing the previously bound small molecules.

CMPD 1 is a hydantoin demonstrating low μM activity in the original HTS campaign which measured the ability of small molecules to interrupt the LFA-1/ICAM interaction. CMPD 2 has similar properties. CMPD 4 is a small molecule with no activity in the LFA/ICAM screen. Figure 1a shows the direct detection of CMPD 1, CMPD 2, and CMPD 4 at 3.2 μM. A similar analysis of these components, again at 3.2 μM, after incubation with 32 μM I domain and isolation of the polypeptide peak by SEC is shown in Figure 1b.

CMPD1 and CMPD2 showed strong binding, as expected, the inactive control showed no binding. The additional closely eluting component in CMPD2 of the same MW also bound, but was not further investigated. This analysis confirms the binding of the original lead compound and a related hydantoin to the I domain portion of LFA. Note that detection requires the small molecule to be released from the complex and thus shows a reversible binding mode. A panel of 16 compounds having K_d from 0.4 nM to 5 uM all showed binding, while several inactive controls did not (data not shown). The technique can distinguish between those which bind and those that do not, but does not exhibit a strong correlation with K_d, at least as implemented here. In previous investigations of this technique [22], the most significant factor in the amount of binding observed was found to be the relationship between off rate and the time required for SEC isolation of the complex.

Competitive Binding of Small Molecule Inhibitors

A photoaffinity probe analog (CMPD 5) was incubated with LFA-1 I domain. The specific, noncovalent binding of the inhibitor is "captured" by a subsequent irradiation causing the benzophenone function to react with nearby (2.5–3.1 Å reactive distance) extractable hydrogens of the target. The covalent bond thus formed is stable to enzymatic digestion and LC/MS procedures [24]. The attachment occurs at Pro281 in the tryptic peptide FASKPASEFVK (residues 277–287) derived from I domain. To demonstrate a correlation between this specific binding of inhibitor and its ability to interrupt the LFA-1/ICAM interaction the following procedure was developed. LC/MS monitored the +2 charge state of the unlabeled peptide at *m/z* 605 and the +2 charge state of the peptide labeled by CMPD5 at *m/z* 973. The percentage response of this peptide undergoing photoattachment under optimized conditions (6.5%) was established. Several inhibitors were tested for competition. Protection is calculated as a relative reduction in percentage response of the labeled/unlabeled peptide for each inhibitor. Approximately equal amounts of photoprobe and inhibitor were employed at about 10-fold molar excess to the LFA I domain.

$$\text{Pr ihbx} = 1 - \frac{R937\text{ihbx}/R605\text{ihbx} + R937\text{ihbx}}{R937\text{cont}/R605\text{cont} + R937\text{cont}} \quad (\times 100)$$

Where Pr is the calculated fraction of protection, R973 and R605 are the integrated responses for the M + $2H^{+2}$ ions of the labeled and unlabeled peptide when incubated with a potential inhibitor (ihbx) or without inhibitor (cont).

The level of protection for competitive binding to I domain can be compared with K_d values determined from the same set of compounds in the binding assay which measures the ability of these inhibitors to interrupt the binding of LFA-1 to ICAM-1 [5]. The inhibitor K_d's range from 50 nM to 10 μM. A plot of K_d's for inhibition of LFA-1/ICAM binding versus protection of Idomain PA labeling produced a slope of 1.17 and a r^2 of 0.94 (see Figure 2). All data points were used to calculate r^2. This correlation demonstrates that binding to the specific site identified on the I domain is directly responsible for the interruption of ICAM-1/LFA-1 binding. CMPD 5 has a K_d of 460 nM in the LFA-1/ICAM binding assay [5]. That assay has a good deal of uncertainty, typically a factor of 2 to 3. It is also likely that some of CMPD 5 is consumed in side reactions during the irradiation making its effective concentration lower and increasing the level of protection.

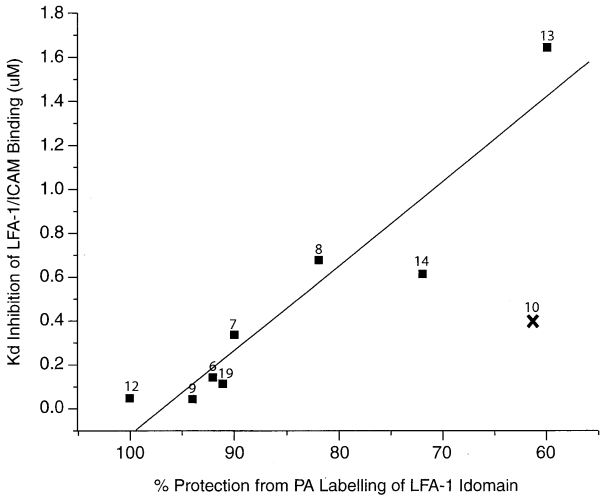

Figure 2. Correlation between inhibition of LFA-1/ICAM binding and protection from photoaffinity labeling of LFA-1 I domain by several hydantoins. x = not used in calculation of the line. Several weak binding inhibitors that did not protect LFA-1 I domain from PA labeling are off-scale and thus not shown.

Direct Observation of Inhibitor Binding to LFA-1 I Domain

Direct ESI was employed to analyze I domain after incubation with CMPD 6 and other inhibitors, at neutral pH in ammonium acetate buffer. Low "skimmer voltage" and ESI source temperature were employed to minimize fragmentation of the noncovalently bound inhibitors. Most of the ion current was contained in the +9 and +8 charge states. LFA-1 has a metal binding site, which is important for its function [26]. The protein preparation contained manganese and this remained bound after the dialysis. The binding of Mn and the ligand are shown to be noncompetitive consistent with the results of PA and modeling [7]. This observation is, of course, only made after the experiment so it was important to maintain Mn bound to the I domain. This somewhat restricted our ability to perform desalting of

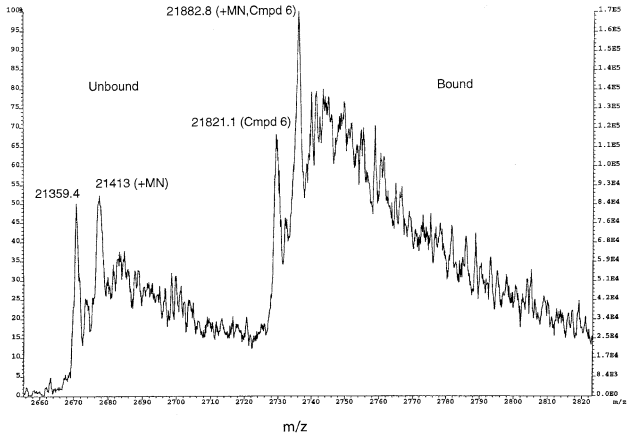

Figure 3. Direct ESI (nondenaturing conditions) of LFA-1 I domain incubated with CMPD 6, expansion of +8 charge state around *m/z* 2700. Calculated molecular weights are shown for identified species.

the protein. In practice this did not prevent us from accurately calculating the molecular weight of the bound small molecule or observing a 1:1 stoichiometry. Figure 3 shows an expansion of the *m/z* region containing the +8 charge state. Ions formed from I domain without CMPD 6 (with and without Mn) were observed. A more abundant pair of ions formed from I domain with bound CMPD 6 (again with and without Mn) was also observed. Only one molecule of inhibitor binding was observed, even with molar excess of the inhibitors. This is evidence for the specificity of the binding. A panel of 12 compounds having K_d's from 1 to 0.01 μM was analyzed in a similar manner. Eleven showed binding. The ratio of bound to unbound peaks varied from compound to compound, but did not correlate with K_d. Since all were present at levels significantly above their K_d, it may be pointed out that their ability to survive ESI without fragmentation did not correlate with K_d. This is consistent with the observation that these K_d's are largely dependent on hydrophobic interactions. The strength of hydrophobic interactions often does not correlate with ESI stability, in contrast to ionic and hydrogen-bonding interactions that often show a strong correlation with ESI stability [27].

Features of the ESI Spectrum Indicate Conformational Change on Small Molecule Binding

The charge state distribution in an electrospray spectrum has been shown to correlate with tertiary protein structure [10–12, 17]. Mass spectra acquired under "nondenaturing" conditions have fewer charges as tertiary folding covers the polypeptide's basic residues. The spectra often have a narrow distribution of charge states consistent with a single, dominant tertiary structure. Observation of a multi-modal distribution is evidence for the presence of more than one structure. In this study the spectrum of I domain was dominated by

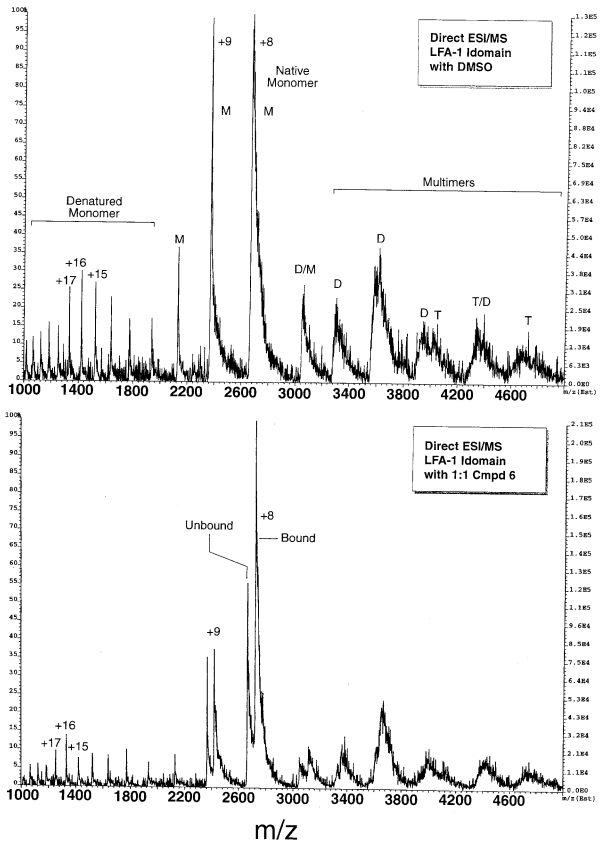

Figure 4. Alteration in ESI spectra of LFA1 I domain produced by compound binding (above) control with no hydantoin, (below) with Compound 6. M = monomer, D = dimer, T = trimer.

ions of +9 and +8 charge states (see Figure 4 a and b). This is consistent with a single predominant tertiary conformation. This distribution shifts toward the +8 charge state on small molecule binding. This is consistent with either an alteration of tertiary structure and/or covering of a protonation site by the inhibitor. A second broader distribution, of lower abundance, from +22 to +13 charge states was also observed. This distribution is similar to that obtained by ESI under denaturing conditions. The relative abundance of these two conformations was found to vary with small molecule binding. Figure 5 shows the variation of peak heights, in each distribution, observed on addition of CMPD 6. The drastic reduction of response from denatured species indicates that a different and stronger conformation is formed on compound binding. This conclusion is valid regardless of whether the denaturation occurs in solution or during the ESI process.

Another set of charge distributions was observed in the ESI spectra due to formation of multimers of the I domain (this is also shown in Figure 5). Multimeric species were observed in the *m/z* range 3000–5000. The formation of these species was suppressed on compound binding. This also suggests an alteration of tertiary structure. Presumably, compound binding produces a more rigid structure without the flexibility

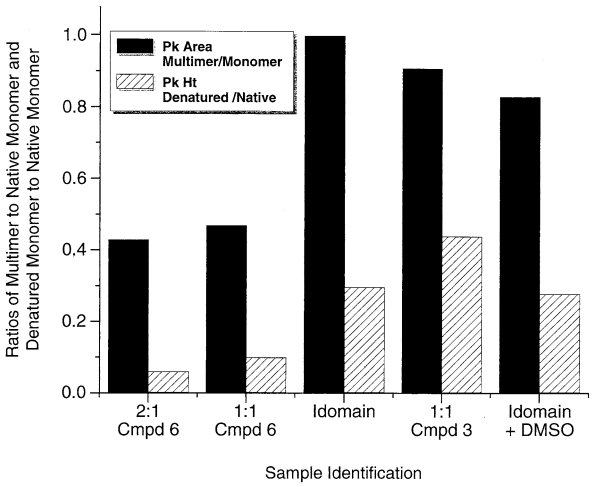

Figure 5. Alterations in the ESI mass spectra of LFA1 I domain (acquired under "native conditions") caused by inhibitor binding. Striped bars are the ratio of peak heights for higher charge states assigned to denatured monomer/peak heights of lower charge states produced by the "native" monomer. Black bars are the ratio of peak areas from charge states due to multimer formation/peak areas from charge states assigned to native monomer.

required to participate in the nonspecific interactions required for multimer formation. ESI Spectra acquired with an inactive hydantoin analog (CMPD 3) did not produce these alterations.

The interaction of LFA-1 with ICAM involves two very large molecules with a large area of contact. In this sense a small molecule should not be able to bind a small pocket and block activity, analogous to blocking the active site of a catalytic enzyme, for instance. The observation of conformational change on compound binding, by ESI MS, is consistent with a mechanism involving the locking of LFA-1 into an inactive conformation unable to bind ICAM.

Conclusion

Mass Spectrometric methods demonstrate that a specific mechanism of binding is responsible for the inhibition of the LFA-1/ICAM-1 interaction by small molecule hydantoins. This reversible binding occurs at a site located within the I domain of LFA-1. Conformational changes of the LFA-1 I domain are indicated, by mass spectrometry, consistent with the ability of a small molecule to prevent the binding of two large proteins having a large surface of interaction. The sensitivity and flexibility of the MS methods allowed important information to be obtained rapidly and efficiently. This supplied direction and impetus to the project, in general, and in particular to characterization of the mode of action of the hydantoin inhibitors.

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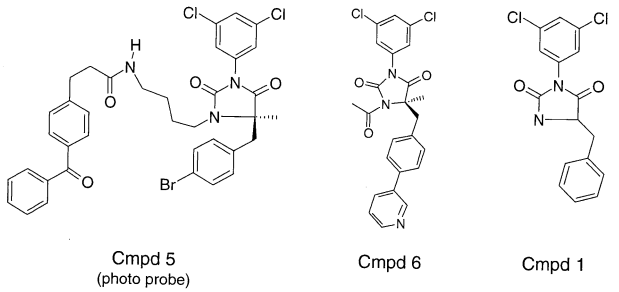

Scheme 1. Structures of representative hydantoins employed in this work.

References

1. Springer, T. A. Adhesion Receptors of the Immune System. *Nature* **1990,** *346,* 425–434.
2. Gahmberg, C. G. Leukocyte Adhesion: CD11/CD18 Integrins and Intercellular Adhesion Molecules. *Curr. Opin. Cell Biol.* **1997,** *9,* 643–650.
3. Vazeux, R. P.; Hoffman, A.; Tomita, J. K.; Dickinson, E. S.; Jasman, R. L.; St. John, T.; Gallatin, W. M. Cloning and Characterization of a New Intercellular Adhesion Molecule. *Nature* **1992,** *360,* 485–488.
4. Dustin, M. L.; Springer, T. A. T-Cell Receptor Cross-Linking Transiently Stimulates Adhesiveness Through LFA-1. *Nature* **1989,** *341,* 619–624.
5. Kelly, T. A.; Jeanfavre, D. D.; McNeil, D. W.; Woska, J. R.; Reilly, P. L.; Mainolfi, E. A.; Kishimoto, K. M.; Nabozny, G. H.; Zinter, R.; Bormann, B. J.; Rothlein, R. A Small Molecule Antagonist of LFA-1 Mediated Cell Adhesion. *J. Immunol.* **1999,** *163,* 5173–5177.
6. Woska, J. R.; Shih, D.; Kelly, T. A.; Taqueti, V. R.; Hogg, N.; Kishimoto, T. K. Antagonist of LFA-1 Blocks a Conformational Change Important for LFA-1 Function. *J. Leukocyte Biol.* **2001,** *70,* 329–334.
7. Last-Barney, K.; Davidson, W.; Cardozo, M.; Frye, L. L.; Grygon, C. A.; Hopkins, J. L.; Jeanfavre, D. D.; Pav, S.; Qian, C.; Stevenson, J. M.; Tong, L.; Zindell, R.; Kelly, T. A. Binding Site Elucidation of Hydantoin Based Antagonists of LFA-1 Using Multidisiplinary Technologies: Evidence for the Allosteric Inhibition of a Protein–Protein Interaction. *J. Am. Chem. Soc.* **2001,** *123,* 5643–5650.
8. Przybylski, M. Mass Spectrometric Approaches to the Characterization of Tertiary and Supramolecular Structures of Biomacromolecules. *Adv. Mass Spectrom.* **1995,** *13,* 257.
9. Loo, J. A. Studying Noncovalent Protein Complexes by Electrospray Ionization Mass Spectrometry. *Mass Spectrom. Rev.* **1997,** *16,* 1–23.
10. Pramanik, B. N.; Bartner, P. L.; Mirza, U. A.; Liu, Y.; Ganguly, A. K. Electrospray Ionization Mass Spectrometry for the Study of Noncovalent Complexes: An Emerging Technology. *J. Mass Spectrom.* **1998,** *33,* 911–920.
11. Veenstra, T. D. Electrospray ionization mass spectrometry in the study of biomolecular non-covalent interactions. *Biophys. Chem.* **1999,** *79,* 63.
12. Li, Y. T.; Hsieh, Y. L.; Henion, J. D.; Ganem, B. Studies on Heme Binding in Myoglobin, Hemoglobin, and Cytochrome c by Ion Spray Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **1993,** *4,* 631.
13. Ganem, B.; Li, Y.; Henion, J. D. Detection of Noncovalent Receptor–Ligand Complexes by Mass Spectrometry. *J. Am. Chem. Soc.* **1991,** *113,* 6294–6296.
14. Nemirovskiv, O. V.; Ramamathan, R.; Gross, M. L. Investigation of Calcium-Induced, Noncovalent Association of Calmod-

ulin with Melittin by Electrospray Ionization Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **1997,** *8,* 809.
15. Chowdhury, S. K.; Katta, V.; Chait, B. T. Probing Conformational Changes in Proteins by Mass Spectrometry. *J. Am. Chem. Soc.* **1990,** *112,* 9012–9013.
16. Winston, R. L.; Fitzgerald, M. C. Mass Spectrometry as a Readout of Protein Structure and Function. *Mass Spectrom. Rev.* **1997,** *16,* 165–179.
17. Light-Wahl, K. J.; Schwartz, B. L.; Smith, R. D. Observation of the Noncovalent Quaternary Associations of Proteins by Electrospray Ionization Mass Spectrometry. *J. Am. Chem. Soc.* **1994,** *116,* 5217–5278.
18. Smith, D. L.; Zang, Z. Probing Noncovalent Structural Features of Proteins by Mass Spectrometry. *Mass Spectrom. Rev.* **1994,** *13,* 411–429.
19. Last, A. M.; Robinson, C. V. Protein Folding and Interactions Revealed by Mass Spectrometry. *Curr. Opin. Chem. Biol.* **1999,** *3,* 564–570.
20. Dunayevskiy, Y. M.; Lai, J.; Quinn, C.; Talley, F.; Vouros, P. Mass Spectrometric Identification of Ligands Selected from Combinatorial Libraries Using Gel Filtration. *Rapid Comm. Mass Spectrom.* **1997,** *11,* 1178–1184.
21. Siegel, M. M.; Tabei, K.; Bebernitz, G. A.; Baum, E. Z. Rapid Methods for Screening Low Molecular Mass Compounds Noncovalently Bound to Proteins Using Size Exclusion and Mass Spectrometry Applied to Inhibitors of Human Cytomegalovirus Protease. *J. Mass Spectrom.* **1998,** *33,* 264–273.
22. Blom, K. F.; Larsen, B. S.; McEwen, C. N. Determining Affinity-Selected Ligands and Estimating Binding Affinities by Online Size Exclusion Chromatography/Liquid Chromatography Mass Spectrometry. *J. Comb. Chem.* **1999,** *1,* 82–90.
23. Moy, F. J.; Haraki, K.; Mobilio, D.; Walker, G.; Powers, R.; Tabei, K.; Tong, H.; Siegel, M. M. MS/NMR: A Structure-Based Approach for Discovering Protein Ligands and for Drug Design by Coupling Size Exclusion Chromatography, Mass Spectrometry, and Nuclear Magnetic Resonance Spectroscopy. *Anal. Chem.* **2001,** *73,* 571–581.
24. Prestwich, G. D.; Dorman, G.; Elliot, J. T.; Marecak, D. M.; Chaudhary, A. Benzophenone Photoprobes for Phosphoinositides, Peptides, and Drugs. *Photochem. Photobiol.* **1997,** *65,* 222–234.
25. Fleming, S. A. Chemical Reagents in Photoaffinity Labeling. *Tetrahedron* **1995,** *51,* 12479–12520.
26. Michishita, M.; Viden, V.; Arnout, M. A. A Novel Cation-Binding Site in the a Domain of Integrin CR3 (CD11b/CD18) is Essential for Ligand Binding. *Cell* **1993,** *72,* 857–867.
27. Smith, R. D.; Bruce, J. E.; Wu, Q.; Lei, P. New Mass Spectrometric Methods for the Study of Noncovalent Associations of Biopolymers. *Chem. Soc. Rev.* **1997,** *26,* 191–202.