Application of Native ESI-MS to Characterize Interactions between Compounds Derived from Fragment-Based Discovery Campaigns and Two Pharmaceutically Relevant Proteins

Originally published in:
SLAS DISCOVERY 23(9), https://doi.org/10.1177/2472555218775921

Application of native ESI-MS to characterize interactions between compounds derived from fragment based discovery campaigns and two pharmaceutically relevant proteins

Agni F. M. Gavriilidou[1], Finn P. Holding[2], Joseph E. Coyle[2], Renato Zenobi[1]

[1]ETH Zurich, Department of Chemistry and Applied Biosciences, CH-8093 Zurich, Switzerland

[2]Astex Pharmaceuticals, 436 Cambridge Science Park, Milton Road, Cambridge, CB4 0QA, United Kingdom

Correspondence to: Renato Zenobi; e-mail: renato.zenobi@org.chem.ethz.ch

Keywords: native mass spectrometry, fragment based drug discovery, noncovalent interactions

Abstract: Native electrospray mass spectrometry (ESI-MS) was applied to analyze binding of compounds generated during fragment based drug discovery (FBDD) campaigns against two functionally distinct proteins the X-linked inhibitor of apoptosis protein (XIAP) and cyclin dependent kinase 2 (CDK2). Compounds of different molecular weight and a wide range of binding affinities obtained from the hits to leads and lead optimization stages of FBDD campaigns were studied, and their dissociation constants (Kd) were measured by native ESI-MS. We demonstrate that native ESI-MS has the potential to be applied to the stages of an FBDD campaign downstream of primary screening for the detection and quantification of protein-ligand binding. Native ESI-MS was used to derive Kd values for compounds binding to XIAP and the dissociation of the complex between XIAP and a peptide derived from the second mitochondria-derived activator of caspases protein (SMAC) induced by one of the test compounds was also investigated. Affinities of compounds binding to CDK2 gave Kd values in the low nM to low

mM range and Kd values generated by MS and ITC followed the same trend for both proteins. Practical considerations for the application of native ESI-MS are discussed in detail.

Introduction.

Fragment-based drug discovery (FBDD)[1–3] is a pharmaceutical approach for generating small molecule inhibitors. It involves the identification of small (< ~350 Da), low-affinity molecular fragments that are subsequently evolved into lead compounds by iterative cycles of medicinal chemistry.

A range of biophysical methods are used in fragment-based drug discovery including nuclear magnetic resonance (NMR), protein X-ray crystallography, surface plasmon resonance (SPR), and thermal shift (T_m) measurement to study weaker interactions. The affinity of protein-ligand interactions during the first stage of a FBDD campaign would typically be in the μM to low tens of mM range and would decrease to nM or sub-nM for a lead compound after the hits-to-leads phase (H2L) and lead optimization (LO) stages. In the H2L phase initial fragment hits are optimized into "lead" compounds that have good potency and other promising properties and in the LO phase a lead compound is further optimized so that the resulting "candidate drug" has all the in vitro and in vivo properties required to make it a suitable candidate for clinical trials. The various techniques to analyze ligand binding often require large amounts of purified protein and compounds, and may also be restricted in their use by factors such as compound solubility[3] and crystallizability. There may also be a requirement for immobilization of the protein on a surface (SPR) or the use of reporter molecules such as dyes (T_m) for fluorescence-based methods, and these can impose their own limitations on the analysis of protein-ligand binding events. Sensitive complementary techniques are therefore required to address these challenges and to support

FBDD campaigns by detecting weak noncovalent binding and generating estimations of dissociation constants (K_d) values. Weak affinity chromatography (WAC) combined with mass spectrometry (MS) is an emerging technology for fragment screening based on selective retention of fragments by a drug[4,5]. Here it is demonstrated that native mass spectrometry can also be applied during a FBDD campaign.

The use of native MS as a primary screening tool has previously been reported, but despite the development of native MS methods for studying noncovalent interactions between proteins and small molecules[6,7], as described in a 2013 review by Poulsen[8], application of native MS to the LO and H2L phases of a FBDD campaign, downstream from the primary fragment screening, has not been widely reported.

Maple et al.[9] described a primary fragment-based screen of 157 compounds by native MS using a TriVersa NanoMate (Advion)[10,11] for automated nanospray infusion of protein-ligand complexes, which was completed and analyzed in 6 h. A significant amount of time was required to achieve the optimal instrument conditions, but it was demonstrated that the throughput of native MS is comparable to NMR or isothermal titration calorimetry (ITC), and required less protein and compound. Native MS could therefore be integrated into a fragment based drug discovery program for both screening and post screen characterization of ligand binding. Woods et al[12] and Drinkwater et al[13] used native MS as a complementary method to X-ray crystallography, SPR and ITC to demonstrate the applicability of native MS as a complementary technique in FBDD.

Native mass spectrometry can preserve weakly bound protein-ligand complexes in the gas phase, it is rapid and sensitive, uses comparatively low amounts of protein and compounds, and requires neither crystallization, derivatization, nor immobilization. It is able to measure dissociation

constants over a wide dynamic range and K_d values in the range of low nM to high mM have previously been reported[14,15].

In this study, the applicability of native MS to analyze binding of early stage, low affinity, fragments as well as higher affinity compounds generated in the H2L and LO stages was investigated, and the key experimental parameters were determined. Compounds discovered by Astex's FBDD platform[16,17] against the X-linked inhibitor of apoptosis protein (XIAP), which regulates the apoptotic response, and cyclin dependent kinase 2 (CDK2), a regulatory element for natural cell progression, were analyzed. The study composed of a small set of compounds, which were hits from primary screens and still cover a wide affinity range and were used to demonstrate the utility of native MS.

Native MS yielded information about the stoichiometry of ligand binding to XIAP, the relative binding strengths of the second mitochondrial activator of caspase (SMAC) consensus peptide and one of the test compounds, and absolute quantification of binding affinities for four noncovalent protein-ligand complexes were also determined. Moreover, the ability of one of the test compounds to disrupt the XIAP-SMAC peptide interaction was analyzed in a competition format. For the purpose of validation, three of the four compounds were also analyzed by ITC for their binding to XIAP and the results were in good agreement with MS results. Eight noncovalent CDK2-compound complexes were also studied and their binding affinities were quantified by native MS. Binding affinities of five of the eight compounds analyzed by MS were also determined by ITC and the K_d values found to be in the range from low nM to low mM. Practical considerations and the limitations for the application of native MS during the H2L and LO stages of a FBDD campaign are discussed.

Experimental

All solvents were purchased from Sigma Aldrich (Buchs, Switzerland). Recombinant CDK2 and XIAP protein, SMAC peptide composed of nine amino acids from the C-terminal and all compounds (Table S1) were obtained from Astex Pharmaceuticals. The protocol for the expression and purification of XIAP is published in Chessari G. et al.[16], and that of CDK2 can be found in the SI.

ITC measurements were performed on a Microcal VP-ITC instrument (MicroCal, Northampton, MA, USA) at 25 ^{o}C. XIAP measurements were done in two solutions, 20 mM ammonium acetate (NH_4Ac), pH 7.5, and 50 mM HEPES, 100 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP), pH 7.5. Both solutions gave similar results, but HEPES was used for the ITC measurements because of its higher buffering capacity. For CDK2 50 mM NH_4Ac, 5% dimethyl sulfoxide (DMSO), pH 7.5 was used for all measurements. All ITC experiments were configured with protein in the sample cell and compound in the injection syringe and data were fitted to a single site binding model using Origin 7.0 software.

Solubility measurements were carried out by 1H quantitative NMR at 500 MHz using the QUANTAS approach[18] with dual suppression (WGATE and PRESAT for the water resonance and PRESAT for the acetate resonance). Aliquots of small molecules (100 mM in DMSO-d6) were diluted twenty-fold with buffer (50 mM NH_4Ac, 15 % D_2O, pH = 7.5) to generate samples with nominal concentrations of 5 mM. A 2.5 mM sample of p-hydroxybenzoic acid, prepared by dilution of a 50 mM stock in DMSO-d6 x 20 with buffer, was used as the qNMR standard. Data were processed using MNova (qNMR plugin) reprocessing software.

MS experiments were carried out under native-like conditions using 20-50 mM NH_4Ac adjusted to pH 7.5. In the case of XIAP 10 mM imidazole was added. Stock protein solutions were

desalted and buffer exchanged against NH_4Ac and stock solutions of the SMAC peptide and compounds were prepared either in NH_4Ac or in DMSO. The final concentration of DMSO in protein-ligand samples for MS analysis was 1 % v/v. The protein concentration was kept constant at 5 μM and the compound concentration was varied from 1 μM to 5000 μM. ESI spectra were acquired with a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF ULTIMA, Waters/Micromass, Manchester, UK) in positive ion mode. Sample solutions were directly infused with gold/palladium-coated borosilicate glass nano ESI emitters (Thermo Fisher Scientific, Reinach, Switzerland) using a commercial nano ESI ion source (Waters/Micromass, Manchester, UK). The operating parameters of the MS were adjusted as follows: capillary voltage = 1.5 kV, the cone and first ion tunnel RF1 voltages = 40 and 30 - 50 V respectively, backing pressure = 0.3 - 0.5 bar, the ion source was maintained at ambient temperature. The hexapole collision cell was filled with argon (purity 5.0, PanGas, Zurich, Switzerland) and the collision energy offset (CE) = 2 - 5 V. The CE was kept low to minimize dissociation of the noncovalent complexes in the gas phase.

To determine K_d values, the experimentally derived relative peak areas were used. It was assumed that the ionization efficiencies for the apoprotein and the complex were equal, which allowed the use of the peak area ratios of the free protein and the complex from the mass spectrum instead of their concentrations. In cases where the compound is small compared to the protein, such that the size and surface properties of the protein and the complex are similar, uniform ionization efficiencies are expected[19]. These conditions apply to the studied complexes, since the mass difference between the protein and the complex with its highest-molecular weight compound is <3.5%. Two methods based on the same binding model were used: the titration approach where the data are fitted to the equation derived by Daniel *et al*.[20]:

$$\frac{I(PL)}{I(P)} = \frac{1}{2}\left(-1 - \frac{[P]_0}{K_d} + \frac{[L]_0}{K_d} + \sqrt{4\frac{[L]_0}{K_d} + \left(\frac{[L]_0}{K_d} - \frac{[P]_0}{K_d} - 1\right)^2}\right)$$

and the direct ESI-MS approach[21], which uses the following equation:

$$K_a = \frac{1}{K_d} = \frac{R}{[L]_0 - \frac{R}{1+R}[P]_0}$$

where R = I(PL)/I(P) as defined above. In both cases, the K_d was measured for each charge state independently as well as for the deconvoluted spectrum. The K_d calculations and the fitting of the titration curves were performed using the MATLAB software (2013a, The MathWorks, Natick, MA).

Results & Discussion

The chemical structures and physicochemical properties of the compounds used in this study are summarized in **Table 1** and S1. In Table S2 the calculated mass difference between the protein-ligand complex and the apoprotein is given.

Mass spectra were obtained for 5 µM XIAP incubated with a ten-fold molar excess of the four compounds **1-4** (**Figure 1**). The peak ratio (R) of the complex (PL) to apoprotein (P), R = I(PL)/I(P), was calculated for each spectrum by integrating the relevant peak areas (I) from all charge states. The R values for XIAP-ligand complexes with the compounds **1-4** were 1.51, 0.85, 0.64, 0.34 respectively, where a higher R reflects a higher binding affinity. As described in a 2010 review by Hannah et al[22], the affinity of test compounds can be ranked in this fashion by comparing the complex peak areas at equimolar concentrations. Native MS can therefore provide a rapid means to rank compound binding affinities. It is noteworthy that even at this high molar ratio of ligand to protein, no nonspecific binding was observed. Nonspecific binding is a

stochastic phenomenon, which is impossible to predict so the optimal protein to ligand ratio must be determined for each individual protein (*vide infra*).

Zinc is required for a native conformation of XIAP and the calculated mass (11904 ± 2 Da) shows that XIAP was fully zinc bound, which proves that during the native MS experiments it remained folded. For each charge state in **Figure 1** a second peak was observed at m/z = 1800 (7+) and m/z = 2100 (6+) which had the same intensity in the presence of each of the compounds and was independent of the compound concentration. The mass difference between these peaks and those of the non-ligand bound protein was 673 Da, which was subsequently shown by HPLC fractionation and MSMS peptide sequencing to be a hexapeptide with the sequence AVPYPQ which is very similar to the high affinity N-terminal consensus sequence of the SMAC protein, AVPI. The peptide was a component of the bacterial growth medium, which co-purified with the (inhibitor of apoptosis) IAP proteins and proved recalcitrant to complete removal despite multiple purification steps. Interestingly, this hexapeptide was also not competed away by high affinity compounds or the SMAC peptide (Figure S1). This is not uncommon in protein-ligand binding experiments, and our data underscore that MS is capable of distinguishing the species that are present and that bind (or do not bind) to the protein investigated. The same approach was taken to compare binding of compounds **5-12** to CDK2, although the range of affinities in this case was so large that the experiment was carried out using different ligand concentrations (data not shown), but this demonstrated the high dynamic range achievable with native MS with the appropriate experimental design. In order to eliminate the possibility of non-specific binding yielding false positive results, control experiments were carried out for XIAP and CDK2 (Figure S2) in which compounds **5** (CDK2 ligand) and **1** (XIAP ligand) did not form complexes with

XIAP and CDK2 respectively even at a P:L ratio of 1:10, and the free compound peak was the predominant species in the low m/z range.

The K_d of compound **7** for CDK2 was calculated from the titration approach to be 80 ± 30 nM (Figure S3), which is in good agreement with the value calculated via the direct approach of 60 ± 20 nM. In order to study noncovalent ligand binding, native MS has the advantage that it provides a direct measure of ligand binding stoichiometry and can rapidly yield relative affinities for ranking compounds[17] as well as being able to determine K_d values.

For K_d values < 100 μM accurate fits for the titration curves were generated. However, at weaker affinities the R values will not change dramatically[23] and in a titration experiment the ligand will not saturate the protein and the binding curve will tend to a straight line, as observed for the titration of the compound **9** to CDK2 (Figure S4). The measured affinity of this complex in 50 mM NH_4Ac, 1 % DMSO, pH 7.5 was 107 ± 17 μM with the titration method whereas with the direct ESI-MS approach it was 81 ± 4 μM. Reliable titration curves could not be generated for the low affinity compounds to CDK2. In Figure S5 it is shown that the complex peak intensity did not change dramatically in the titration experiment of the compounds **10-12** to CDK2. The direct approach can be applied to estimate the affinity of both weak and strong-binding compounds more rapidly compared to the titration method. Multiple concentrations can be used to directly estimate the binding affinities as long as nonspecific binding is not observed at increased ligand concentration. Precision and accuracy will decrease with lower affinity binding, so data quality should be assessed for each ligand and a titration experiment used when the data quality is insufficient for a single point determination. As the direct ESI-MS and titration methods resulted in similar K_d values, the direct ESI-MS approach was used for the estimation and ranking of the affinities of the remaining compounds (**Table 1**).

Of the compounds for which K_d values were determined by MS, five of the eight CDK2 compounds and three of the four XIAP compounds, were also benchmarked against K_d values determined by ITC (**Figures 4** and S6). ITC experiments could not be done for all the compounds due to limited protein supply and/or compound insolubility highlighting the key benefit of native MS that it requires comparatively low quantities of protein. ITC data for the lowest and highest K_d values that could be confidently measured before compound solubility issues compromised these measurements (e.g. compound **11**) are shown in **Figure 2** and MS and ITC values for XIAP and CDK2 both showed the same trend. There were small discrepancies which may be due to ion suppression by non-volatile salts in the MS experiments, the presence of impurities and high concentrations of the test compound. But it is important to note the potential limitations of both techniques: ion suppression may adversely affect the accuracy of MS whereas compound solubility may prove limiting in ITC. For accurate affinity measurements by MS the intensity of the peaks should be proportional to the concentration of the analyte in solution[24,25]. However, where protein supply or compound solubility are limiting for ITC, the results of these experiments demonstrate that native MS can rapidly provide K_d values and/or affinity ranking.

When designing native MS experiments for determination of accurate binding affinity values it is essential to optimize the following factors: i) compound concentration - the exact concentration of the protein and the compound as well as the solubility of the compound must be known to reliably measure affinities, ii) solvent - a volatile buffer suitable for ESI-MS must be used to ensure protein stability and compound solubility, iii) MS parameters - they require fine control in order to minimize gas-phase dissociation of weak protein-ligand complexes and simultaneously

desolvate and desorb adducts, and iv) protein/ligand ratio – must be controlled to minimize nonspecific binding of the ligand to the protein.

(i) Compound concentration. DMSO is used routinely for fragment solubilization and storage prior to use in a screening experiment. The final sample will therefore contain a small percentage of DMSO, typically 1-5 %, so the effect of DMSO on CDK2 was investigated (Figure S7). DMSO concentrations up to 5% were tested for the effect on charge state distribution and a decrease in the number of charge states was observed at higher DMSO concentration suggesting compaction of the protein which is in agreement with previously published observations[26]. DMSO concentrations of <10 % result in narrow charge distributions, however more charging is observed at higher DMSO concentrations indicating that supercharging may be causing the protein to unfold and this may alter the binding affinity of noncovalent complexes[27]. It is therefore critical that the DMSO concentration is kept as low as possible.

Poor solubility of some fragments may render accurate concentration determination difficult even in the presence of a high percentage of DMSO, for instance, compound **6** used in this study was poorly soluble (< 10 μM, Table S1). In order to ensure accurate determination of ligand concentrations the solubility of all the CDK2 test compounds was first determined by NMR solubility measurements in the MS buffer, 50 mM NH_4Ac, 1 % DMSO, pH 7.5 and the results are shown in Table S1. In order to quantify affinities of proteins and ligands with any analytical technique including ESI-MS, the concentrations of both the protein and the compounds should be known accurately. Therefore, solubility measurements are a requisite part of the experimental workflow.

(ii) ESI compatible buffer. In order to detect weakly bound compounds the signal-to-noise (S/N) ratio of the protein-ligand complex was optimized (Figure S8). ESI is sensitive to nonvolatile

solvent additives such as buffer salts, therefore protein solutions are electrosprayed from a solvent that differs from the original purification or storage buffer. Consequently, an effective desalting step is critical to ensure the minimum level of adduction of the protein by nonvolatile buffer components[28]. In this study, small protein sample volumes were desalted by centrifugal gel filtration using Micro Bio-Spin P-6 Gel Columns (BIORAD, Cressier, Switzerland) into NH_4Ac, or by centrifugal filtration (Amicon Ultra-0.5 10K, Merck Millipore, Darmstadt, Germany) with up to 3 cycles of dilution and concentration to exchange the nonvolatile buffer for NH_4Ac.

It has been demonstrated previously[29] that addition of imidazole at high concentration to the nano ESI solution can increase the stability of gas-phase complexes, therefore representing the solution phase species more accurately. Imidazole acts as a nonspecific, sacrificial, ligand which prevents dissociation of the specifically bound ligand by enhanced evaporative cooling of the complex. A final concentration of 10 mM imidazole was added to XIAP-ligand complexes, which resulted in an increase in the relative abundance of the complex ions and higher S/N (Figure S9). No difference was observed in the intensities of the CDK2-ligand ion peaks in the presence of imidazole, which shows that each protein has to be individually optimized for native MS experiments.

(iii) MS parameters - collisional dissociation. The collision energy was optimized to minimize dissociation of weak complexes in the gas phase as this would lead to artificially high K_d values and false negative results, which would not be representative of the solution phase. An example of a false negative result in which complete dissociation of the protein-ligand complex was observed can be seen in **Figure 3** (top panel). Conversely, insufficient collision energy led to

incomplete desolvation, and a broad adduct peak distribution, which rendered the data uninterpretable (**Figure 3**, bottom panel).

Collision-induced dissociation (CID) experiments were performed with XIAP to investigate the stability of the noncovalent XIAP-compound complexes in the gas phase. The collision energy offset was varied until the selected parent ions of the noncovalent complexes were completely dissociated and the optimum collision energy to study XIAP complexes was determined from a plot of the normalized percentage of the intact complex against the collision energy offset (Figure S10).

(iv) Nonspecific protein-ligand interactions. High concentrations of compounds may lead to nonspecific interactions with the target protein (**Figure 4**). According to the ES model[30], the initial ESI droplets undergo solvent evaporation until the Rayleigh limit is reached, at which point they undergo fission, releasing several multiply charged droplets containing none, one, or multiple molecules of analyte. The probability of a droplet containing more than one analyte molecule is increased at higher analyte concentration therefore the occurrence of nonspecific binding would also be expected to increase in that instance. For weak binders high concentration of compounds is used, therefore the probability of nonspecific interactions occurring is increased. In this experiment nonspecific binding was clearly observed when the compound was at a thirty-fold molar excess with regard to protein, but was reduced at a twenty-fold molar excess of compound and was eliminated at a ten-fold molar excess. A mathematical approach has been developed[31] to correct the calculation of K_d values in cases where nonspecific binding occurs, which yielded K_d values similar to those derived in solution-phase experiments. Additionally, solution phase competition experiments with a higher affinity tool compound monitored by ESI–MS can discriminate specific from nonspecific interactions[22].

Native MS experiments can also be configured in a competition format which has the advantage that it can directly visualize the replacement of one ligand with a competitive ligand via the observed mass difference between the various protein-ligand species. The influence of test compounds **1** and **2** on the XIAP-SMAC peptide complex was observed using native nano ESI-MS. Compound **1** had the highest affinity for XIAP and was shown to cause dissociation of the SMAC peptide. At higher concentrations of compound, the intensity of the peak corresponding to the XIAP-SMAC peptide complex (PS) decreased, with a concomitant increase in intensity of the XIAP-compound peak, PL (**Figure 5**). The observations for compound **1** provided evidence for the competitive nature of ligand binding in this case. The intensity of the PL peak also increased with higher concentrations of compound **2** which had lower affinity for XIAP, but the change in intensity of the PS peak was less when compared to compound **1**. Another consequence of the reduced affinity of compound **2** for XIAP was manifested in preferential binding of compound **2** to free XIAP protein as it is less able to overcome the binding energy of the PS complex. The observed pattern of displacement of the SMAC peptide was thus consistent with the relative binding affinities of these two compounds.

Various techniques are applied in the course of a FBDD campaign to analyze large number of fragments and related molecules derived from successive cycles of medicinal chemistry. Each of these techniques is subject to its own limitations such as high protein consumption due to low compound binding affinity and/or solubility, or a requirement for labeling or immobilization. Native MS is a rapid and sensitive technique and can provide a direct measure of ligand binding stoichiometry and affinity for ranking compounds. The use of native MS as an orthogonal screen for hit generation by the detection of weak noncovalent binding fragments has already been demonstrated previously[9] but it can also serve as a powerful tool to complement the other stages

of the FBDD process[7]. In this study the aim was to investigate the extended applicability of the technique not just to fragments, but to include higher affinity compounds generated in the H2L and LO stages of two Astex FBDD campaigns against CDK2 and XIAP.

The conditions required to generate reliable binding data for complexes of XIAP and CDK2 with FBDD derived compounds, which possess a broad range of affinities were tested. These included compound solubility, ESI buffer compatibility, MS parameters and the protein/ligand ratio.

The data indicated that with careful experimental design native MS can reliably detect noncovalent protein-ligand binding enabling determination of K_d values and ranking of relative binding affinities over a wide dynamic range. In an FBDD environment, multiple factors influence the design of the experimental approach[2,3] and an important consideration is the availability of the relevant protein, which may be limited and thereby restrict application of techniques such as X-ray crystallography, NMR and ITC, which require large amounts of protein. Native MS can help to address this issue, but each protein target must be individually evaluated for its suitability for MS based ligand binding experiments. The protein must, however, be stable in MS solvent over the timeframe of the experiment, and the effect of DMSO containing buffers on the protein and its complexes must be determined. Experimental conditions should be configured to ensure the native conformation is retained, which is competent to form a complex with low affinity ligands that do not dissociate in the gas phase. The experimental procedure must remove adducts in order that weak-ligand binding with low abundance can be discerned from spectral noise, and ligand binding to low molecular weight compounds can be sufficiently resolved from the apoprotein peak. Sample preparation and analysis is comparatively rapid and thus all the proposed optimization steps may be achieved within an appropriate time frame in the context of a drug discovery project.

Current MS technology is capable of analyzing protein-protein interactions, including those involving membrane proteins[32,33], and whilst analysis of fragments binding to these proteins may not yet be possible, it is conceivable that fragment binding may be detectable using native MS methods in the future. To further augment the analytical platform available for compounds derived from in the H2L and LO phases of FBDD campaigns, we show here that native nano ESI-MS is a technique which can be applied to generate better understanding of protein-ligand interactions, especially when protein availability, compound solubility and other factors may be limiting for other techniques.

ACKNOWLEDGMENT

We are grateful to Dr. Jing Dong for his assistance in protein production and Dr. Torren Peakman for performing the NMR experiments to determine the compound solubility. We thank the Swiss National Science Foundation (grants no. 200020_140398 and 200020_159929) for financial support of this research. All original data, evaluations, plots, etc. will be stored in a curated data archive at ETH Zurich (https://www.research-collection.ethz.ch) under the DOI xxx.

REFERENCES

1 Arkin M. R., Wells J. A. Small-Molecule Inhibitors of Protein–protein Interactions: Progressing towards the Dream. *Nat. Rev. Drug Discov.* **2004**, *3*, 301-317.

2 Scott D. E., Coyne A. G., Hudson S. A., et al. Fragment-Based Approaches in Drug Discovery and Chemical Biology. *Biochemistry* **2012**, *51*, 4990-5003.

3 Murray C. W., Rees D. C. The Rise of Fragment-Based Drug Discovery. *Nat. Chem.* **2009**, *1*, 187-192.

4 Meiby E., Knapp S., Elkins J. M., et al. Fragment Screening of Cyclin G-Associated Kinase by Weak Affinity Chromatography. *Anal Bioanal Chem* **2012**, 2417-2425.

5 Duong-Thi M.-D., Meiby E., Bergström M., et al. Weak Affinity Chromatography as a New Approach for Fragment Screening in Drug Discovery. *Anal Biochem* **2011**, 138-146.

6 Loo J. A. Electrospray Ionization Mass Spectrometry: A Technology for Studying Noncovalent Macromolecular Complexes. *Int. J. Mass Spectrom.* **2000**, *200*, 175-186.

7 Hofstadler S. A., Sannes-Lowery K. A. Applications of ESI-MS in Drug Discovery: Interrogation of Noncovalent Complexes. *Nat. Rev. Drug Discov.* **2006**, *5*, 585-595.

8 Poulsen S. A. Fragment Screening by Native State Mass Spectrometry. *Aust. J. Chem.* **2013**, *66*, 1495-1501.

9 Maple H. J., Garlish R. A., Rigau-Roca L., et al. Automated Protein–Ligand Interaction Screening by Mass Spectrometry. *J. Med. Chem.* **2012**, *55*, 837-851.

10 Zhang S., Van Pelt C. K., Wilson D. B.. Quantitative Determination of Noncovalent Binding Interactions Using Automated Nanoelectrospray Mass Spectrometry. *Anal. Chem.* **2003**, *75*, 3010-3018.

11 Keetch C. A., Hernández H., Sterling A., et al. Use of a Microchip Device Coupled with Mass Spectrometry for Ligand Screening of a Multi-Protein Target. *Anal. Chem.* **2003**, *75*, 4937-4941.

12 Woods L. A., Dolezal O., Ren B., et al. Native State Mass Spectrometry, Surface Plasmon Resonance, and X □ Ray Crystallography Correlate Strongly as a Fragment Screening Combination. *J. Med. Chem.* **2016**, *59*, 2192-2204.

13 Drinkwater N., Vu H., Lovell K. M., et al. Fragment-Based Screening by X-Ray Crystallography, MS and Isothermal Titration Calorimetry to Identify PNMT (Phenylethanolamine N-Methyltransferase) Inhibitors. *Biochem. J.* **2010**, *431*, 51-61.

14 Cubrilovic D., Biela A., Sielaff F., et al. Quantifying Protein-Ligand Binding Constants Using Electrospray Ionization Mass Spectrometry: A Systematic Binding Affinity Study of a Series of Hydrophobically Modified Trypsin Inhibitors. *J. Am. Soc. Mass Spectrom.* **2012**, *23*, 1768-1777.

15 El-Hawiet A., Kitova E. N., Liu L., et al. Quantifying Labile Protein–Ligand Interactions Using Electrospray Ionization Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 1893-1899.

16 Chessari G., Buck I. M., Day J. E. H., et al. Fragment-Based Drug Discovery Targeting Inhibitor of Apoptosis Proteins: Discovery of a Non-Alanine Lead Series with Dual Activity Against cIAP1 and XIAP. *J. Med. Chem.* **2015**, *58*, 6574-6588.

17 Wyatt P. G., Woodhead A. J., Berdini V., et al. Identification of N-(4-Piperidinyl)-4-(2,6-Dichlorobenzoylamino)-1 H-Pyrazole-3-Carboxamide (AT7519), a Novel Cyclin Dependent Kinase Inhibitor Using Fragment-Based X-Ray Crystallography and Structure Based Drug Design. *J. Med. Chem.* **2008**, *51*, 4986-4999.

18 Farrant R. D., Hollerton J. C., Lynn S. M., et al. NMR Quantification Using an Artificial Signal. *Magn. Reson. Chem.* **2010**, *48*, 753-762.

19 Peschke M., Verkerk U. H., Kebarle P.. Features of the ESI Mechanism That Affect the Observation of Multiply Charged Noncovalent Protein Complexes and the Determination of the Association Constant by the Titration Method. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1424-1434.

20 Daniel J. M., Friess S. D., Rajagopalan S., et al. Quantitative Determination of Noncovalent Binding Interactions Using Soft Ionization Mass Spectrometry. *Int. J. Mass Spectrom.* **2002**, *216*, 1-27.

21 Liu L., Kitova E. N., Klassen J. S. Quantifying Protein-Fatty Acid Interactions Using Electrospray Ionization Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 310-318.

22 Vivat Hannah V., Atmanene C., Zeyer D., et al. Native MS: An ’ESI, Way to Support Structure- and Fragment-Based Drug Discovery. *Future Med. Chem.* **2010**, *2*, 35-50.

23 Kitova E. N., El-Hawiet A., Klassen J. S.. Screening Carbohydrate Libraries for Protein Interactions Using the Direct ESI-MS Assay. Applications to Libraries of Unknown Concentration. *J. Am. Soc. Mass Spectrom.* **2014**, *25*, 1908-1916.

24 Root K., Wittwer Y., Barylyuk K., et al. Insight into Signal Response of Protein Ions in Native ESI-MS from the Analysis of Model Mixtures of Covalently Linked Protein Oligomers. *J. Am. Soc. Mass Spectrom.* **2017**, *28*, 1863-1875.

25 Kebarle P., Verkerk U. H.. Electrospray: From Ions in Solution to Ions in the Gas Phase, What We Know Now. *Mass Spectrom. Rev.* **2009**, *28*, 898-917.

26 Sterling H. J., Prell J. S., Cassou C. A., et al. Protein Conformation and Supercharging

with DMSO from Aqueous Solution. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 1178-1186.

27 Cubrilovic D., Zenobi R.. Influence of Dimehylsulfoxide on Protein–Ligand Binding Affinities. *Anal. Chem.* **2013**, *85*, 2724-2730.

28 Hernández H., Robinson C. V. Determining the Stoichiometry and Interactions of Macromolecular Assemblies from Mass Spectrometry. *Nat. Protoc.* **2007**, *2*, 715-726.

29 Sun J., Kitova E. N., Klassen J. S. Method for Stabilizing Protein-Ligand Complexes in Nanoelectrospray Ionization Mass Spectrometry. *Anal. Chem.* **2007**, *79*, 416-425.

30 Dole M., Mack L. L., Hines R. L., et al. Molecular Beams of Macroions. *J Chem.Phys.* **1968**, *49*, 2240-2249.

31 Shimon L., Sharon M., Horovitz A. A Method for Removing Effects of Nonspecific Binding on the Distribution of Binding Stoichiometries: Application to Mass Spectroscopy Data. *Biophys. J.* **2010**, *99*, 1645-1649.

32 Laganowsky A., Reading E., Hopper J. T. S., et al. Mass Spectrometry of Intact Membrane Protein Complexes. *Nat. Protoc.* **2013**, *8*, 639-651.

33 Hopper J. T. S., Yu Y. T.-C., Li D., et al. Detergent-Free Mass Spectrometry of Membrane Protein Complexes. *Nat. Methods* **2013**, *10*, 1206-1208.

Table 1. Compounds, thermodynamic parameters and the calculated dissociation constant K_d with ITC, the direct and titration MS approach for complexes of XIAP with compounds 1-4 and CDK2 with compounds 5-12.

Protein Target & Compounds		ΔH (ITC) (kcal/mol)	-TΔS (ITC) (kcal/mol)	K_d (ITC) (μM)	Direct approach (MS) K_d (μM)	Titration approach (MS) K_d (μM)
XIAP	**1**	-6.6	-2.3	0.3	2.3±0.2	-
	2	-4.7	-2.2	9.0	13±0.6	-
	3	-6.8	1.1	66	67±3	-
	4	-	-	-	142±5	-
CDK2	**5**	-19.3	9.4	0.06	0.04±0.02	0.04±0.01
	6	-	-	-	55±8	-
	7	-15.9	7.1	0.33	0.06±0.02	0.08±0.03
	8	-	-	-	51±10	46±7
	9	-7.9	2.9	250	81±4	107±17
	10	-7.2	2.8	590	1170±272	-
	11	-	-	-	2350±427	-
	12	-6.5	2.1	560	3400±1150	-

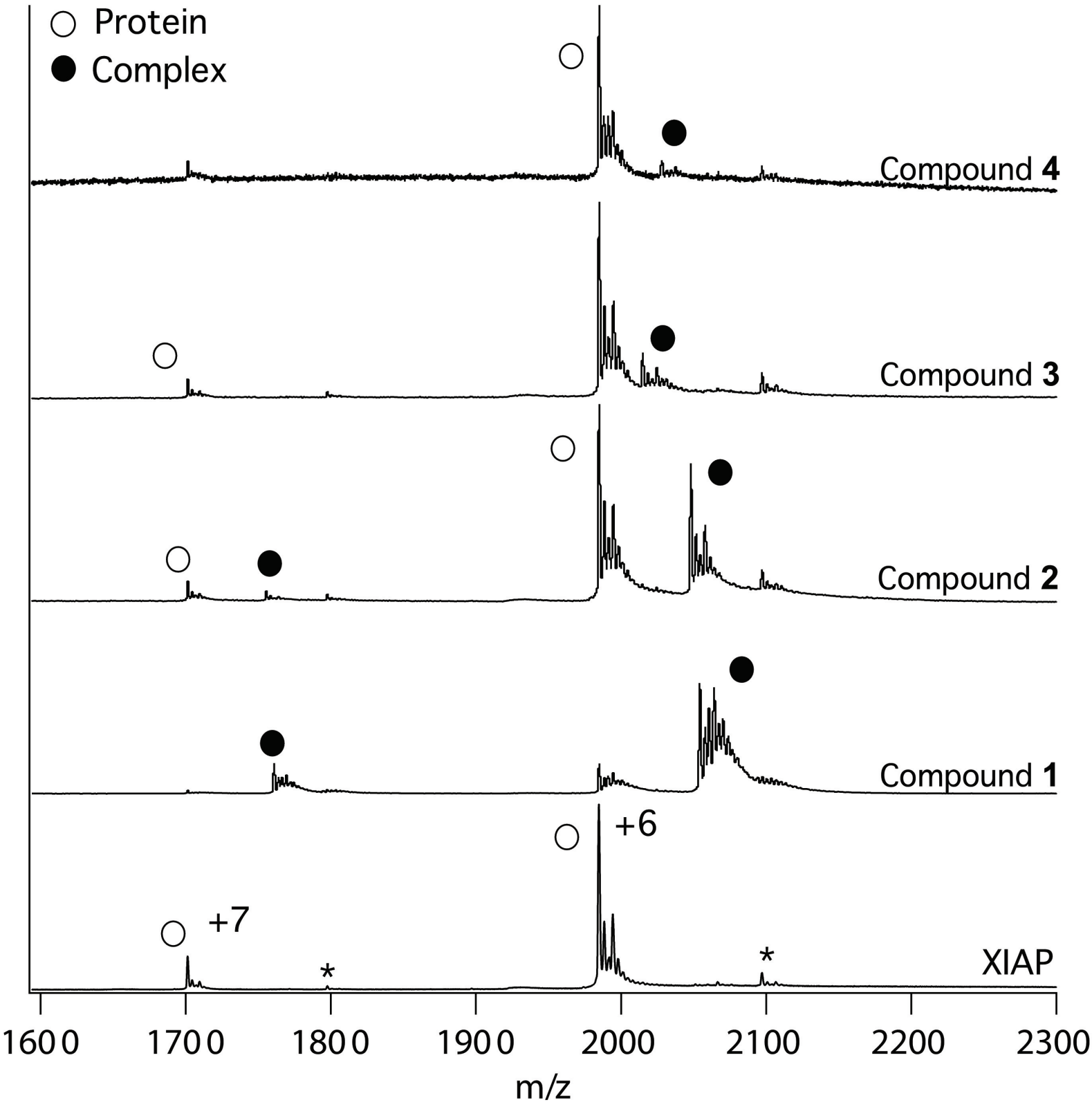
Figure 1: Representative nano ESI-MS spectra of 5 μM XIAP in complex with 50 μM compounds **1-4** in 20 mM NH_4Ac, 10 mM imidazole, pH 7.5. The tighter binding compounds yielded more intense complex peaks. The highlighted peak (*) corresponds to the complex of XIAP with a 673 Da peptide that is a component of the bacterial growth medium.

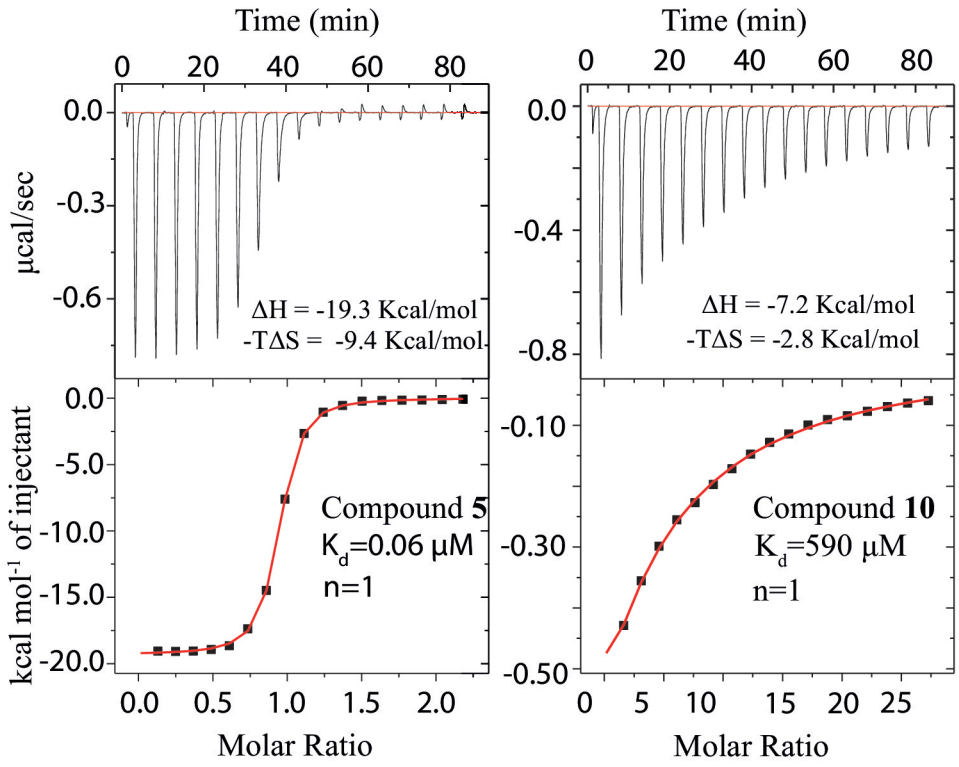
Figure 2: Measurement of thermodynamic parameters including dissociation constant (K_d) and stoichiometry (n) by ITC for interactions between CDK2 with compounds **5** and **10**.

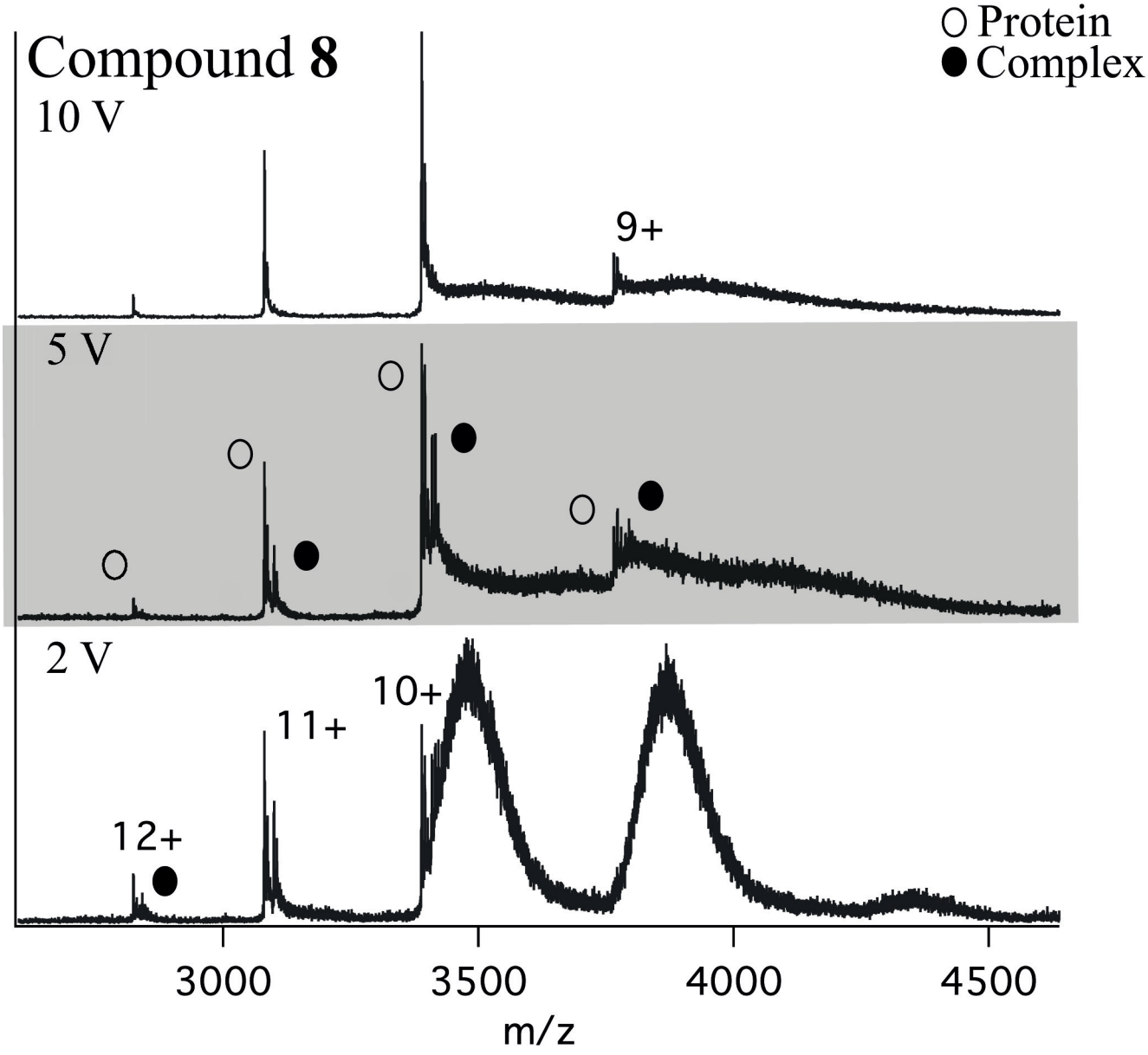
Figure 3: Native ESI-MS spectra of noncovalent complex of 60 μM compound **8** with 5 μM CDK2 at 2-10 V collision energy. Instrument conditions were controlled in order to minimize gas-phase dissociation of the complex and at the same time ensure acceptable levels of desolvation. The spectrum with the optimum collision energy of 5 V is highlighted.

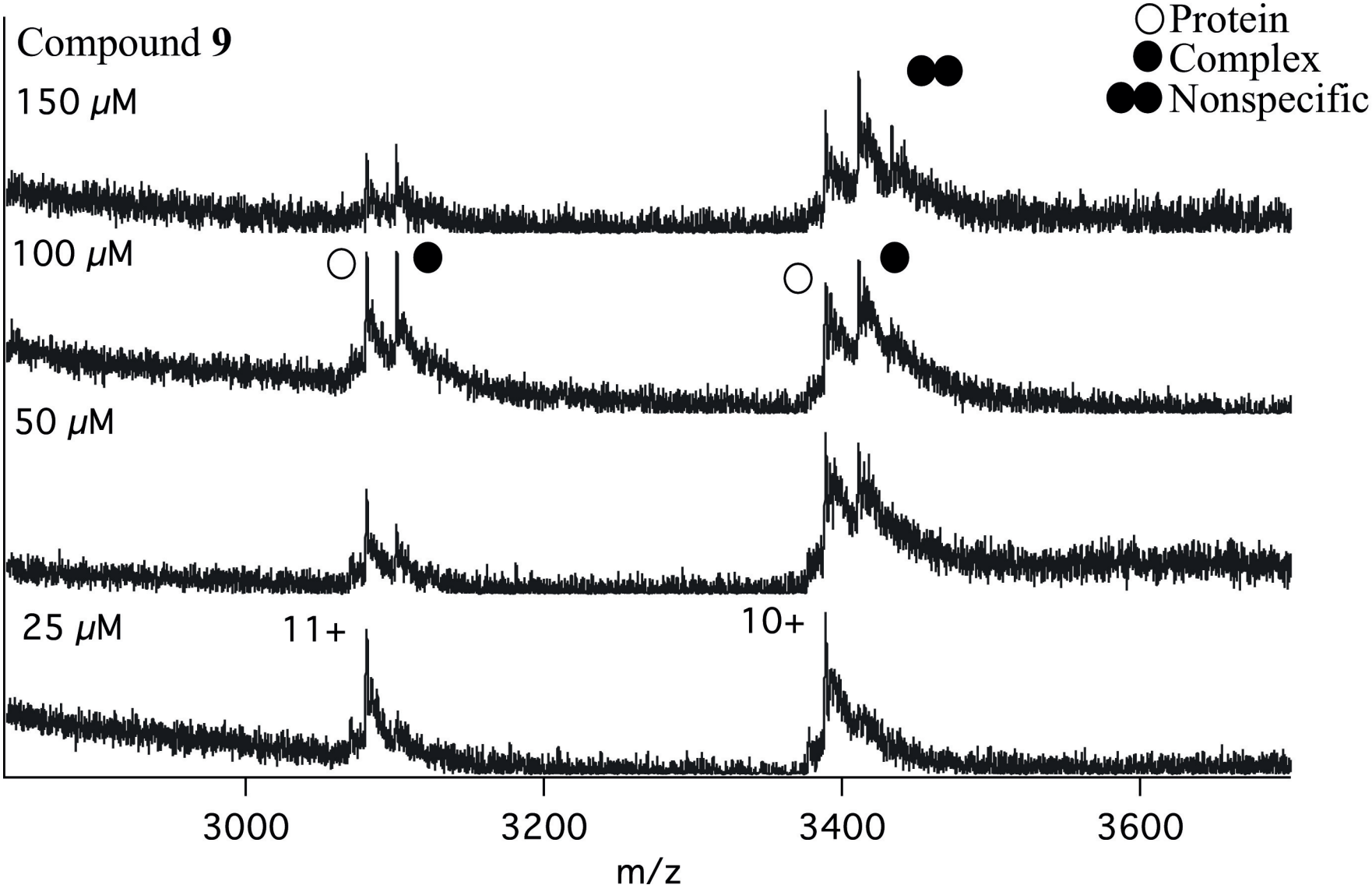
Figure 4: Native ESI-MS spectra of titration of compound **9** against 5 μM CDK2 in 50 mM NH_4Ac, 1 % DMSO, pH 7.5.

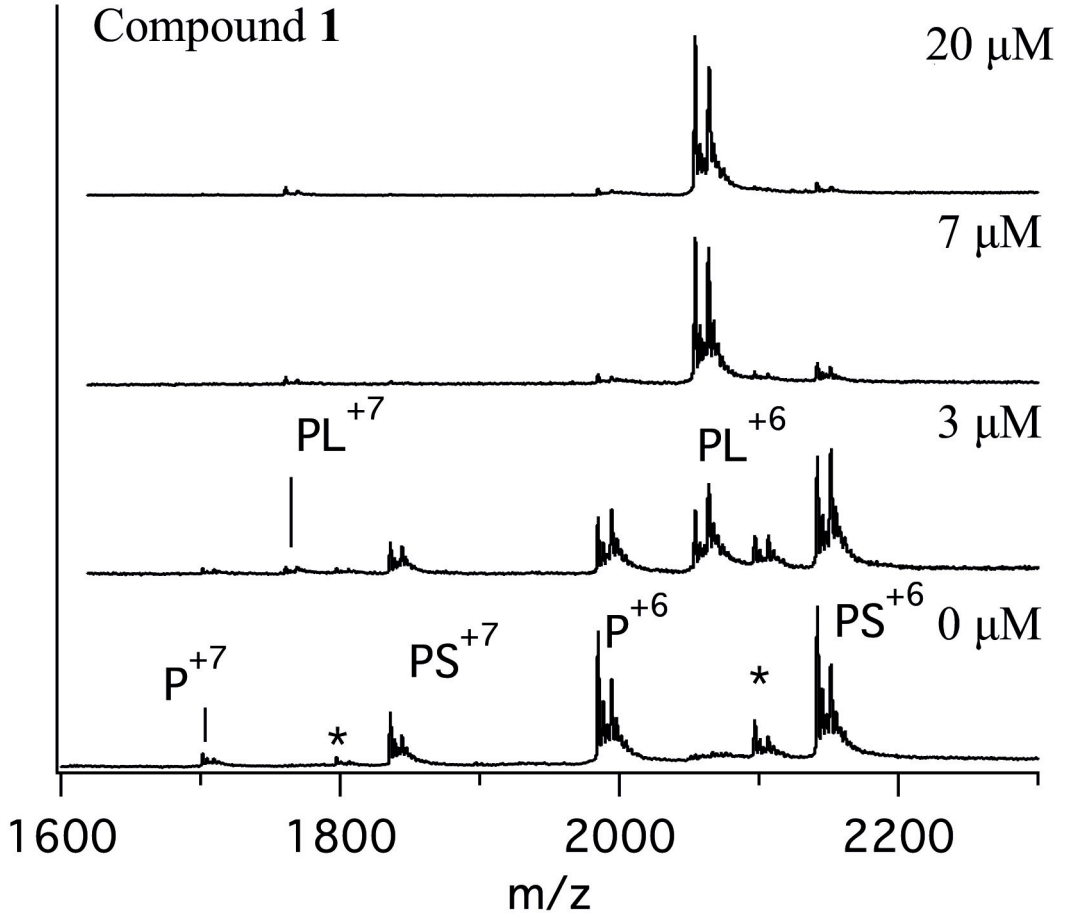
Figure 5. Nano ESI mass spectra of XIAP-SMAC peptide complexes (PS) in the presence of different concentrations of the compounds (L). The PS signal clearly decreased with increasing concentration of compound **1**. Although the intensity of the XIAP-compound (PL) peak increased in the presence of compound **2** the change in intensity of the PS peak was less noticeable than for **1**. The highlighted peak (*) corresponds to the complex of XIAP with a 673 Da peptide that is a component of the bacterial growth medium.

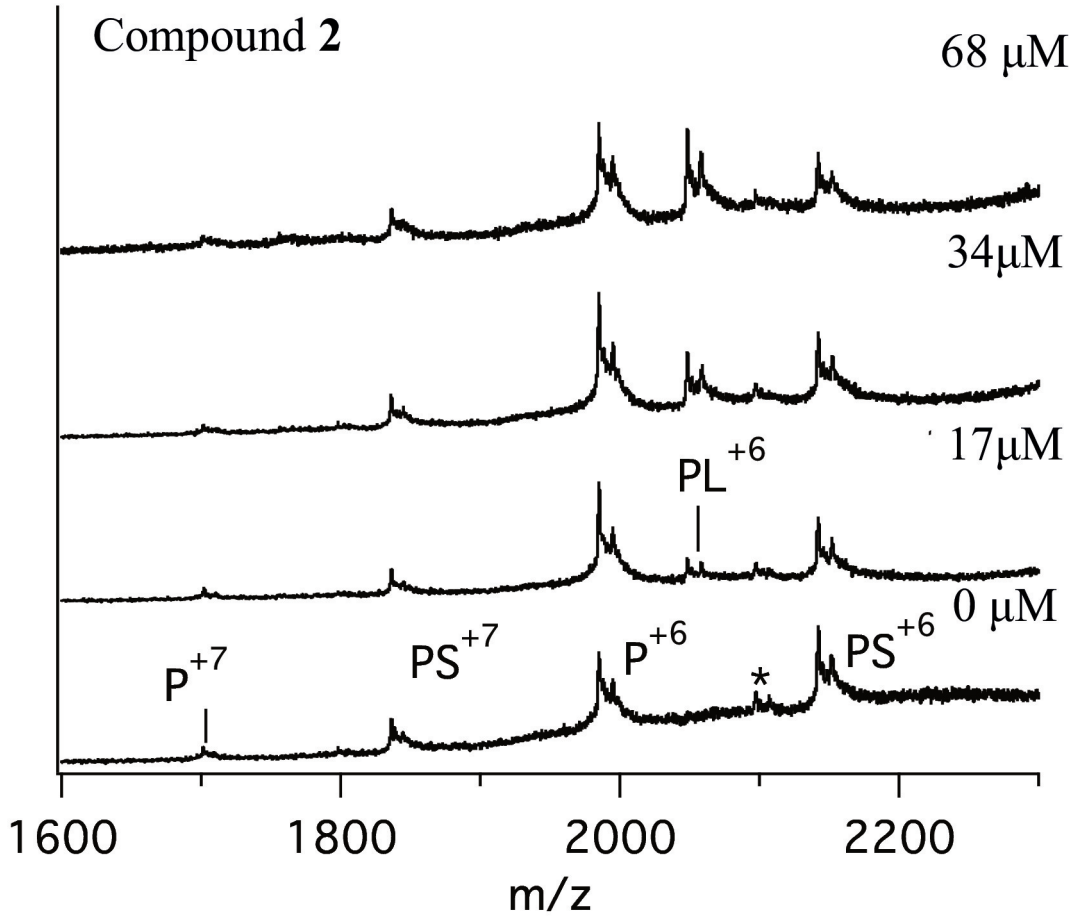

Protein
Complex
Compound 4
Compound 3
Compound 2
Compound 1
XIAP
+6
+7
*
*
1600
1700
1800
1900
2000
2100
2200
2300
m/z


Time (min)
0
20
40
60
80
0.0
-0.3
-0.6
µcal/sec
ΔH = -19.3 Kcal/mol
-TΔS = -9.4 Kcal/mol
0.0
-5.0
-10.0
-15.0
-20.0
kcal mol-1 of injectant
Compound 5
Kd=0.06 µM
n=1
0.0
0.5
1.0
1.5
2.0
Molar Ratio
Time (min)
0
20
40
60
80
0.0
-0.4
-0.8
ΔH = -7.2 Kcal/mol
-TΔS = -2.8 Kcal/mol
-0.10
-0.30
-0.50
Compound 10
Kd=590 µM
n=1
0
5
10
15
20
25
Molar Ratio


Compound 8
10 V
5 V
2 V
Protein
Complex
9+
10+
11+
12+
3000
3500
4000
4500
m/z


Compound 9
150 μM
100 μM
50 μM
25 μM
Protein
Complex
Nonspecific
11+
10+
3000
3200
3400
3600
m/z


Compound 1
20 μM
7 μM
3 μM
0 μM
PL+7
PL+6
P+7
PS+7
P+6
PS+6
*
*
1600
1800
2000
2200
m/z


Compound 2
68 μM
34μM
17μM
0 μM
PL+6
P+7
PS+7
P+6
PS+6
*
1600
1800
2000
2200
m/z