**Understanding Protein-Drug Interactions using Ion Mobility-Mass Spectrometry**

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**Abstract**

Ion mobility-mass spectrometry (IM-MS) is an important addition to the analytical toolbox for the structural evaluation of proteins, and is enhancing many areas of biophysical analysis. Disease-associated proteins, including enzymes such as protein kinases, transcription factors *exemplified by* p53, and intrinsically disordered proteins, including those prone to aggregation, are all amenable to structural analysis by IM-MS analysis. In this review we discuss how this powerful technique can be used to understand protein conformational dynamics and aggregation pathways, and in particular, the effect that small molecules, including clinically-relevant drugs, play in these processes. We also present examples of how IM-MS can be used as a relatively rapid screening strategy to evaluate the mechanisms and conformation-driven aspects of protein:ligand interactions.

**Introduction**

An important consideration during drug development is the structural and mechanistic evaluation of the protein target, ideally combined with a multi-level understanding of how conformation and biological function are modulated by ligand binding. Ion mobility-mass spectrometry (IM-MS), which separates ions in the gas-phase based on their size (mass), shape and charge [1-7] has emerged as an important addition to more traditional structural biology techniques such as NMR, X-ray crystallography and Cryo-electron microscopy [8] and can be readily exploited to help understand conformational dynamics of proteins and non-covalent protein complexes [9-11]. Although IM-MS is unable to reveal resolution at the atomic level, the ability to analyse heterogeneous complexes and protein-ligand interactions in their native conformations [11-19] offers a competitive advantage over other structural approaches, which either ‘fix’ the conformation *e.g.* during crystal formation, or are unable to handle mixtures. Indeed, the fact that analyte mass to charge *(m/z)* ratio is evaluated independently of ion mobility information means that IM-MS can be used to analyse heterogeneous populations; it also provides a means of analysing protein complexes that occupy multiple conformations, whilst providing important information on the stoichiometry of non-covalent complexes. Moreover, application of IM-MS for structural interrogation is typically much faster than other approaches, and only requires picomole amounts of material for analysis. This method can thus be exploited as a stand-alone tool for protein structural interrogation, with or without *in silico* molecular modelling, or to complement high-resolution information acquired by other means. [20] For example, crystallographic evaluation of proteins (with or without bound ligands), particular those with disordered regions, often results in incomplete atomic structures.[21] Combining partial structural datasets with experimentally derived CCS information can therefore be used to constrain topological models through computational approaches. Coarse-grained and homology modelling has proven useful in this regard, being applied to structural modelling of numerous multimeric protein complexes with distinct topologies.[22-25]

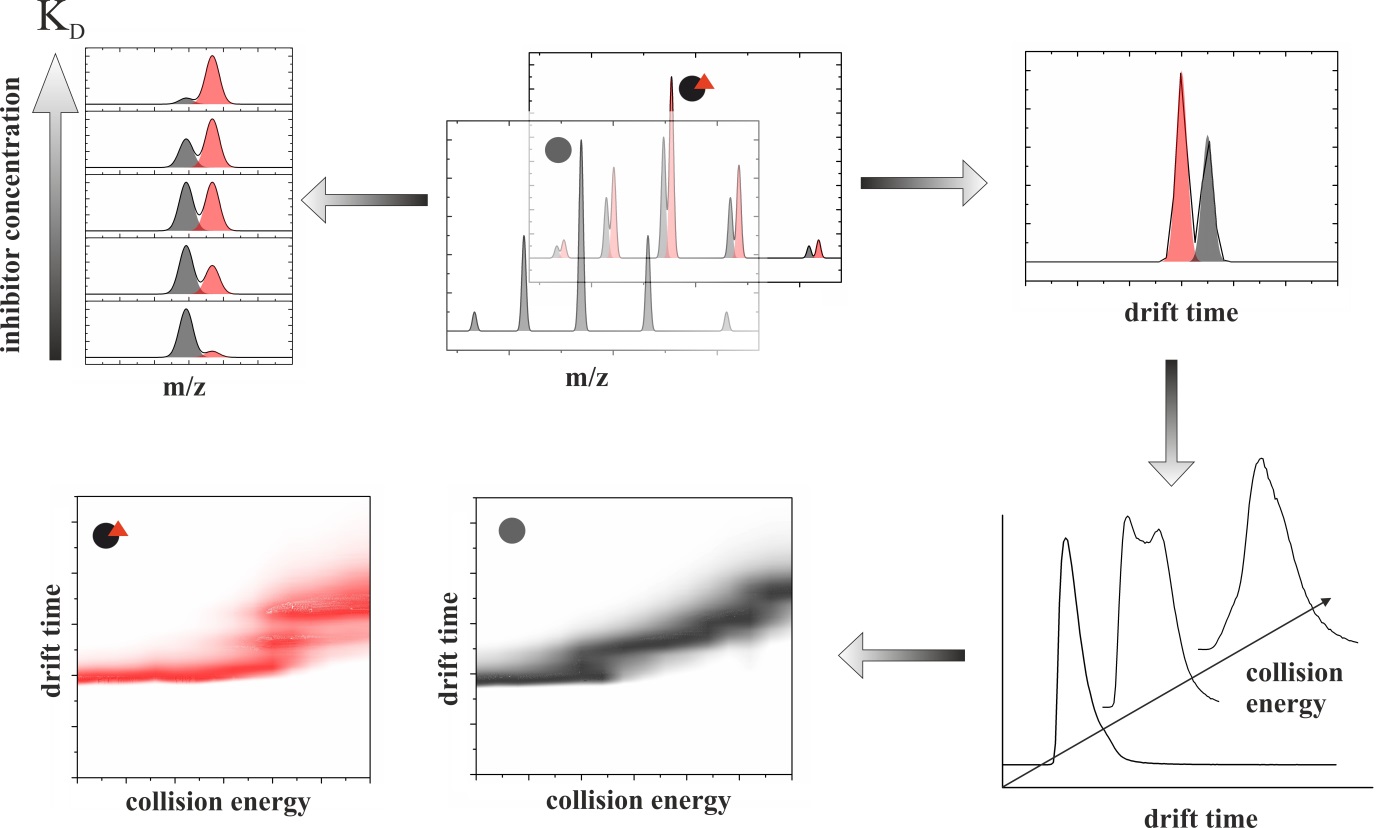
Although originally the subject of some debate, a significant body of evidence now demonstrates that in the majority of cases, the native solution-phase structure of a protein/protein:ligand complex can be retained in the gas phase [26,27], especially when “native” ESI conditions are employed and analytical parameters are carefully controlled. Once in the gas-phase, the resulting ions can be separated based on two physical properties: their differential mobility through an inert gas in a weak electric field, [28] and by employing standard *m/z-*based separation using mass spectrometry (MS). The major purpose of this review is to describe how IM-MS has been applied to help understand different protein-drug interactions, rather than to provide a background to the different forms of this technique. Several comprehensive reviews detailing the fundamentals of IM-MS are available [1,2,6,29]. Of most relevance to this article are drift tube IMS (DTIMS) [3,30], trapped ion mobility IMS (TIMS) [31] and travelling wave IMS (TWIMS) [32], all of which are available on commercial instruments and can therefore be readily employed experimentally. These are discussed briefly below.

DTIMS employs a weak homogeneous electric field to direct ions through a drift cell, where collisions between ions and an inert buffer gas (typically helium or nitrogen) delay passage through the cell, allowing the rotationally averaged collisional cross section (CCS) of different ions to be measured directly. Unlike DTIMS, which directly assesses the drift time of ions in a ‘stationary’ gas, in TIMS, ions are trapped in the presence of a counter-flow of gas, with ions ‘eluting’ selectively according to their relative mobility through the gas. TWIMS is comparable to DTIMS, but uses a stacked ring ion guide (SRIG) through which ions are propelled by means of a travelling wave DC voltage superimposed onto a radially-confining RF voltage. Ions of high mobility will spend less time in the travelling wave ion guide (TWIG) than ions of low mobility as they will be more easily transported through the gas-filled mobility cell. Calibration of the time taken for ions of known CCS to travel through the TWIG can then be used to compute CCS values of unknown analytes of similar chemical structure under the same buffer and voltage conditions [33-36]. CCS reports median protein conformation, while concurrent measurement of the CCS distributions (CCSD) can be used to help evaluate conformational flexibility. Moreover, when experimental CCS information is compared with theoretical calculations for a given geometry, possible candidate structures can be proposed, ruled out or validated.Field asymmetric IMS (FAIMS), also called differential IMS, is an alternative method for separating gaseous ions at atmospheric pressure[37,38]. However, due to the non-linear effect of the applied electric field on ion mobility and its deleterious effect on native protein conformation, FAIMS cannot be used to determine CCS, but is instead used to separate mixtures. However, as with all IM-MS instrumentation, FAIMS can be exploited to determine the dissociation constant (KD) of protein:ligand complexes, although this type of IMS is optimal for stronger non-covalent complexes. IM-MS derived KD values are thought to be comparable with values obtained using more traditional solution approaches, such as fluorimetry, calorimetry, thermophoresis and Surface Plasmon Resonance (SPR).

Depending on the type of IM-MS employed, structural information pertaining to protein:ligand complexes can thus be obtained, including definition of KD for reversible binding, and by providing insight into whether ligands stabilize (or destabilize) protein conformations (see Figure 1). KD values are determined using the titration method followed by a nonlinear curve fit using Equation 1 [39]. I(PL) and I(P) define the peak area of the protein:ligand complex and the unbound protein respectively; [P]0 and [L]0 are the original protein and ligand concentrations:

Equation 1 

The effect of ligand binding on protein conformational stability can also be investigated using collision-induced unfolding (CIU) [40-42]. Here, the intact protein:ligand complex (or the remaining unbound protein) is subjected to a gradual increase in collisional activation below that required for protein or complex dissociation, thereby inducing protein unfolding. The (partially) unfolded protein states at different activation energies are then analysed by IM-MS, with the resultant ‘CIU fingerprint’ being used to define the partially unfolded transition states as a function of the applied energy (see Figure 1). Software packages *e.g.* CIUSuite, can assist with data interpretation [43] and, in the case of ORIGAMI, also automate acquisition of CIU fingerprints [44].



*Figure 1: Native IM-MS can yield information regarding the effect of ligand binding on protein conformation and stability. Ion mobility is used to resolve possible conformational changes. Acquiring arrival time distributions (ATDs) at different collision energies results in a CIU fingerprint and allows information to be obtained about ligand binding strength as well as conformational stability of the bound or unbound protein. Acquisition of mass spectra at different small molecule or ligand concentrations can be used to determine the strength of interaction by calculation of a dissociation constant KD.*

As exemplars of the utility of IM-MS for structural characterisation of protein-ligand interactions, we will focus on three major classes of protein for which IM-MS has revealed important information: amyloid, intrinsically disordered proteins (IDPs) and protein kinases.

**Amyloid**

Protein aggregation and the formation of amyloid fibrils is thought to be a causative factor in over 50 human diseases including Alzheimer’s disease [45], Parkinson’s disease [46], type II diabetes [47], cardiovascular disease, and some forms of cancer [48]. IM-MS has been used to provide important insights into the self-assembly mechanisms of several distinct amyloidogenic proteins, including 2-microglobulin, A40/A42 and -synuclein [49,50], helping to understand the assembly and architecture of fibrils and associated intermediates, and the effect of small molecules on these dynamic processes (Figure 2) [51-55]. More recent work by Pagel and colleagues used a combination of infrared spectroscopy with IM-MS to directly analyse the secondary structure of individual amyloid intermediates, elegantly demonstrating that small fibril-forming 6-mer peptides yield oligomers comprising an extensive β-sheet architecture. [56]

In contrast to other structural techniques, IM-MS can characterise the multiple individual soluble aggregate forms present during the transition from monomers to insoluble fibrils, as opposed to an ‘average’ of properties forming an oligomer ensemble (Figure 2). One of the first studies demonstrating the applicability of IM-MS to amyloidosis was an analysis of early aggregation states of A42, a putative neurotoxic species in Alzheimer’s disease [57]. The results of this investigation were in agreement with labour-intensive photochemical cross-linking experiments, and further supported by molecular modelling. A follow-on study revealed that the mechanism of A42 aggregation is different to that of A40, and demonstrated that the Aβ42 dodecamer is likely to be the primary toxic species in Alzheimer’s disease [51].

*Figure 2: Schematic illustrating IM-MS monitoring of the self-assembly process of different amyloid structures in the absence and presence of an inhibitor. (Top panels) Cartoon mobilograms of amyloid proteins in the absence (left) and presence of an inhibitor of amyloidosis (right). The number of amyloid subunits, n, is determined by MS analysis, while the collision cross section (conformation) of the oligomers is determined using IMS. (Bottom panels) The mechanism proposed by Bleiholder et al [52] suggests that monomers self-assemble leading to the formation of soluble oligomers (n=2-4), at which point there is a transition from a globular conformation to a presumed toxic linear β-sheet structure. In the presence of inhibitors of amyloidosis, aggregates either do not form (not shown) or are thought to maintain an isotropic globular conformation (n≥4, bottom right).*

Crucial for the development of therapeutic strategies for amyloid-triggered disease is understanding the aggregation process in the presence of peptide or small molecule-based ligands (Figure 2) [58-65]. A classic example is the study by Ashcroft, Radford and colleagues, who demonstrated the utility of IM-MS for screening a panel of small molecules for disrupting amyloid formation of human islet amyloid polypeptide (hIAPP) and Aβ40 [66,67]. By developing a high throughput screen, several parameters could be assessed simultaneously: protein:ligand interactions; the protein species (monomer, oligomer) to which the drug bound; whether binding was specific or non-specific and the effect of ligand binding on self-assembly pathways. Ultimately, this led to the identification of novel inhibitors of *in vitro* Aβ40 fibril formation*.*

By combining IM-MS with complementary strategies such as electron-transfer dissociation (ETD) and molecular modelling, additional insight into the mode of ligand binding and the behaviour of specific amino acids in the target can be obtained for observed aggregates [68,69]. Indeed, in combination with NMR and molecular dynamics, IM-MS has recently been used to define the mechanism of binding of the small molecule A2 aggregation inhibitor tramiprosate, which is currently in phase III clinical trials for Alzheimer’s disease [70].

**Intrinsically Disordered Proteins**

Intrinsically disordered proteins (IDPs), which include amyloidogenic proteins such as α-synuclein, lack a well-defined tertiary structure and are believed to adopt a variety of different conformations, including several regions that are predicted to be totally unstructured [71]. IDPs account for a significant percentage of all proteins, with some 40% of human polypeptides predicted to contain at least one disordered region. Moreover, ordered:disordered transitions commonly occur during endogenous protein folding subsequent to translation [72]. Understanding the structured regions and degree of (dis)order of this important class of proteins is currently restricted, due to the limitations of traditional techniques such as X-Ray crystallography, which require a fixed, relatively stable structural protein disposition in order to form crystals. Due to their roles in forming protein complexes implicated in disease, IDPs have become valuable new drug targets. Consequently, defining changes in structure and conformation that occur upon drug binding can reveal important information about the mode of action of potential therapeutic compounds. The ability of IM-MS to evaluate conformational changes in heterogeneous mixtures with very poorly defined (or absent) higher-order structure thus makes it an ideal technique for IDP interrogation, although its application in this important area is currently underexploited. An important example is the intrinsically disordered transcription factor p53 [73], a tumour suppressor with a short half-life in cells due to its interaction with the Ubiquitin E3-ligase MDM2, which targets p53 for degradation [74,75]. The dysregulation of p53 in many cancers (and stabilisation upon DNA damage) means that understanding, and potentially disrupting, the p53:MDM2 complex, is a desirable chemotherapeutic strategy that has become an exemplar in the protein:protein interaction field [76]. Using native IM-MS, Dickinson *et al.* evaluated the effect of two small molecules, RITA and Nutelin-3, which were designed to interfere with the formation of the p53:MDM2 complex by binding to either p53 or MDM2 respectively [73]. The non-covalent RITA/p53 complex was unstable under the relatively benign conditions used in these analyses, suggesting a lower KD than previously measured in solution, or a requirement for hydrophobic interactions that are lost upon transfer to the gas-phase. However, by evaluating the variance in charge state distribution and CCSD in the absence or presence of RITA, a compound-dependent conformation change in p53 with a modest decrease in protein disorder and compaction, was observed. Similarly, conformational changes were also reported in the N-terminal region of MDM2 in the presence of the high-affinity (KD ~100 nM) small molecule ligand Nutlin-3, which was observed to bind preferentially to a more compact (and relatively inflexible) form of MDM2 [73]. Based on these important observations relating conformation to binding, it is clear that IM-MS has much to offer in the field of IDPs and protein:protein interactions, and their stabilisation/destabilisation by small molecules.

**Protein Kinases**

Protein kinases regulate dynamic protein phosphorylation, which is essential for cell signalling, but also dis-regulated in diseases such as cancer and diabetes. Protein kinases usually cycle between a variety of distinct conformers, presenting numerous opportunities for ligand-binding and conformational trapping. Consequently the human protein kinase superfamily (the kinome) has become a highly valuable class of structurally-distinct targets for therapeutic intervention. Conservation of the protein kinase catalytic domain, in particular the ATP-binding site, means that it is a challenge to selectively modulate specific members of the kinome in this region. Indeed, even after three decades of intensive study, ongoing work still seeks to categorize binding mechanisms of protein kinase inhibitors to help explain conformation-dependent effects in cells. The seminal discovery of kinase inhibitors that induce distinct structural effects on the same target [77] and the synthesis of both allosteric and covalent kinase small molecule inhibitors [78] and activators [79], reinforces the need for technologies to dissect ligand-dependent allosteric communication [80] and conformation-dependent effects of ligands on kinases.

One of the earliest studies using IM-MS to investigate ligand binding to protein kinases studied interaction of the archetypical protein kinase A (PKA) regulatory subunit with analogues of cAMP [81]. Using IM-MS, the antagonistic R-enantiomer of thio-substituted cAMP (RpcAMPS) preferentially bound and stabilised the ‘H-form’ of PKA, while the S-enantiomer agonist SpcAMPS was shown to bind the same conformational state (the ‘B-form’) as the natural ligand cAMP [81]. These studies pave the way for design of novel allosteric PKA R-subunit inhibitors, with a goal of regulating interactions with the PKA catalytic subunit and modulation of the cellular response to G-protein coupled receptors (GPCRs). A separate study employing native MS reports analysis of a pentameric PKA holoenzyme (2 regulatory:2 catalytic subunits) bound to a fragment of the A-kinase anchoring protein AKAP79 under physiological cAMP conditions [82]. It will thus be interesting to evaluate the conformational dynamics (CCSD) of these subunits after selective release from the pentamer, or additionally in the presence of small molecule kinase inhibitors.

In an unrelated study on the same PKA signalling system components, the PKA inhibitor protein (PKI), a very potent endogenous protein inhibitor of the PKA catalytic subunit (PKAc), was shown by IM-MS to exhibit preferential binding to the more compact catalytically-active form of PKAc associated with C-subunit autophosphorylation (Figure 3) [83]. Interestingly, CIU studies revealed that the PKI-binding conformer of PKAc exhibited greater structural stability than the PKAc conformer unable to bind to PKI, which was itself much more similar to deposphorylated PKAc. IM-MS with CIU, and complementary thermal stability studies employing differential scanning fluorimetry (DSF), further demonstrated the effects of PKAc hyper-phosphorylation and conformation-dependent binding of a panel of small molecule kinase inhibitors (including both classical PKA inhibitors such as H89 and the clinical phase I oral multi-AGC kinase inhibitor AT13148). Interestingly, the PKAc:drug complexes exhibited markedly reduced conformational flexibility, confirming that significant structural changes occur upon binding to different classes of small molecule. In another related study, IM-MS and enzyme-based strategies were exploited to demonstrate that the atypical two component hexameric histidine kinase ExsG can exist in both a phosphorylated but catalytically inactive ‘compact’ conformation, and an ‘open’ catalytically active conformation that is associated with nucleotide *binding. [84]*

*Figure 3: Conformation-dependent binding of PKI to PKA catalytic domain (PKAc). Mass spectra (left), ion mobility spectra (middle) and CIU plots (right) of PKAc in the absence (top) and presence (bottom) of full length PKI protein. PKAc/PKI in the mass spectrum (bottom left) refers to the non-covalent protein kinase:inhibitor complex. IM-MS of PKAc demonstrates co-existence of two primary conformations: a more compact conformer able to bind PKI (shaded red), and amore elongated conformer that in unable to bind PKI, termed PKAc(PKI), (shaded green). CIU plots (right) demonstrate a significant difference in the relative stability of the total PKAc conformer population compared to the non PKI-binding kinase conformation [PKAc/(PKI)] as demonstrated by the collision voltage required to induce protein unfolding (white dotted line). See Ref [83] for further information.*

Finally, a key study evaluating drug binding to protein kinases employed a CIU-based IM-MS assay to analyse the model tyrosine protein kinase ABL in the presence of a panel of protein kinase inhibitors [85]. Compounds were categorised as either “type 1” (ATP-competitive) or allosteric “type 2” inhibitors. Although only 11 protein kinase inhibitors were screened, the scalability of such assays theoretically permits the analysis of hundreds of small molecules per day, using relatively small quantities of enzyme. Furthermore, by undertaking dose-dependent evaluation of ligand binding, KD values might also be determined with relative ease, circumventing issues associated with analysis of clinically-relevant inactive and pseudokinase conformations found across the kinase superfamily [80,86-90].

**Future Prospects**

Although not yet fully integrated into the arsenal of tools for structural interrogation of protein complexes, several studies have now demonstrated the power of IM-MS and CIU, often in combination with other biophysical techniques, for characterising protein: ligand interactions that are of importance to both basic and pharmaceutical research. Since these procedures can be adapted for low to medium-throughput screening of ligands, they can be readily integrated into drug discovery and target validation pipelines, and will be particularly useful where mechanistic understanding is lacking. However, while IM-MS is a unique “conformation-based” small molecule screening tool that can be used to probe the strength of protein:ligand interactions in the form of KD value determination, it fails to provide the empirical atomic level detail essential for true target-based drug-design.

In this review, we have focused on clinically relevant protein classes, where better understanding of small molecule binding on protein stability and conformation can reveal insights into therapeutic target development. However, the principles of structural analysis using IM-MS are relevant to protein-ligand binding in any field. It is worth noting that the resolving power of ion mobility separation is still a potential limiting factor, with minor conformational differences (<1%) arising upon small molecule binding especially challenging to define. The commercialisation of a cyclic drift tube in which ions can travel for a variable (increasing) number of cycles before they are ejected, could circumvent this issue [5,91-93]. Indeed, this type of experimental setup has been demonstrated to increase resolving power up to 20-fold, albeit not yet with proteins, which could in the future permit evaluation of extremely small ligand-driven protein conformational changes.

As with any assay, consideration should also be given to the possibility of non-specific ligand binding, which can be assessed by varying buffer conditions used for ‘native’ electrospray ionisation (ESI). Excess ligand or the addition of essential buffer components (*e.g.* salt) can also suppress ESI, resulting in MS spectra of reduced resolution, with poor signal to noise [94,95]. Differential protein ionisation in the absence or presence of ligand, which can sometimes arise due to preferential ionisation of the small molecule, can induce a shift in the ESI charge state envelope, making comparison of the CCS/CCSD for analogous charge states of the bound and unbound protein forms problematic. Dissociation of some weak non-covalent binders that are dependent on hydrophobic interactions may also be lost during transfer to the gas-phase, potentially leading to underestimation of KD [96]. Here, even more gentle evaporation processes like cold spray ionization [97], in conjunction with a low temperature drift tube [98,99] could possibly improve confidence in structural elucidation and KD determination.

IM-MS has enormous potential for the development and screening of ligands, from low molecular weight compounds to therapeutic antibodies, which is only now beginning to be realised. Furthermore, since it is also possible to evaluate binding to differentially modified protein forms (proteoforms) and membrane proteins [19] that might otherwise be intractable to structural interrogation, IM-MS opens up the possibility of understanding the intricacies of small molecule binding to complex proteoforms, such as differentially phosphorylated or glycosylated protein targets. As well as the small ligands discussed here, IM-MS has demonstrated utility in the evaluation of structural differences in antibodies following drug conjugation, a growth area in the pharmaceutical industry [100]. Structural interrogation of protein:DNA and protein:RNA complexes, to ascertain sequence-specific binding affinity, is also likely to benefit from IM-MS analysis [101,102]. Moreover, with the development of robotic chip based injection systems [103] combined with already available automated open-source software for data extraction, processing and visualisation (*e.g.* Pulsar[19], Amphitrite [104], ORIGAMI [44] and UniDec [105]), the age of high throughput IM-MS-based ligand screening appears to have arrived.

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