Strategies for the NMR-Based Identification and Optimization of Allosteric Protein Kinase Inhibitors

Wolfgang Jahnke,* Marcel J. J. Blommers, César Fernández, Christian Zwingelstein, and René Amstutz[a]

Dedicated to Prof. Dr. Horst Kessler on the occasion of his 65th birthday

Protein kinases are important drug targets, but kinase inhibitors ought to be selective and specific in order to avoid side effects in the clinic. Kinase inhibitors that do not target the highly conserved ATP-binding site, but that target an allosteric site, are generally expected to be more selective for the target kinase and thus have a better clinical profile. Here we propose an NMR-based strategy to discover and optimize allosteric kinase inhibitors. The approach uses a spin-labeled adenine analogue to detect allosteric kinase ligands by paramagnetic relaxation enhancement.

Protein kinases comprise a large family of enzymes that catalyze the transfer of the terminal phosphate from ATP (adenosine triphosphate) to protein substrates, specifically to the hydroxyl group of serine or threonine (Ser/Thr kinases) or tyrosine (Tyr kinases). Protein kinases play a crucial role in signal transduction and thereby regulate central cellular processes such as cell-cycle control, growth control, apoptosis, and transcriptional activation.[1] Kinase activity is generally tightly regulated, but can get out of control with overactive or constitutionally activated kinases. Several pathological states or diseases, such as cancer, can be a consequence of kinase overactivation. Small molecules that can modulate kinase activity in vivo are therefore of high therapeutic interest, and those kinases with a central and specific role in a particular disease are pharmaceutically highly relevant drug targets.[2,3] A recent example of successful target selection and inhibitor design is the clinical success of Gleevec®, a low-molecular-weight inhibitor of the constitutionally activated tyrosine kinase, Bcr-Abl.[4]

Protein kinases generally consist of a catalytic (SH1) domain and one or several regulatory (e.g. SH2 or SH3) domains. The catalytic domains have a conserved three-dimensional fold with a bilobed structure: an N-terminal lobe consisting mainly of β sheets and a C-terminal helical lobe (Figure 1). The catalytic site is located near a hinge region that connects these two domains.[5] Kinases can adopt multiple conformational states that are associated with the degree of catalytic activity: fully active kinases are generally phosphorylated in their activation loop, which adopts a conformation that allows for optimal binding of ATP/Mg^{2+} and substrate protein, and for efficient transfer of the phosphate group of ATP. There are several regulatory mechanisms by which a kinase becomes down-regulated or "inactive". The conformational consequence of kinase down-regulation can be movement of the activation loop or other components so that the substrate cannot be efficiently bound to the kinase catalytic domain.[6] Besides the ATP-binding site and the substrate binding site, allosteric binding sites occur in kinases, often at sites with regulatory control function.[6–9]

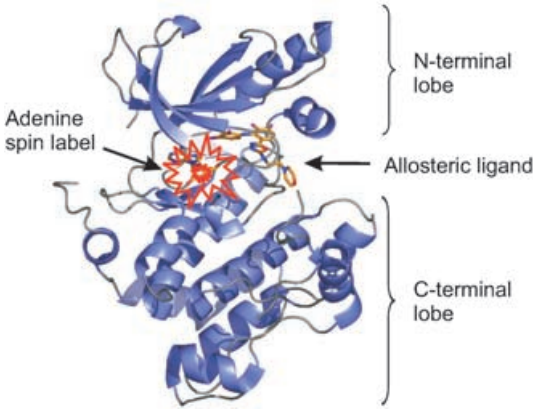

Figure 1. Principle of the experiment. Spin-labeled adenine analogue **3** is bound to the ATP-binding site. Any ligand that binds simultaneously but at a different binding site feels the paramagnetic relaxation enhancement mediated by **3**. The structure of MEK2 in complex with PD334581 and ATP[9] is shown here solely to illustrate the technique.

More than 500 kinases are estimated to be encoded in the human genome. All of them bind ATP/Mg^{2+}, and the ATP-binding site is highly conserved both in amino acid sequence and in three-dimensional structure. Kinase inhibitors that target the ATP site in an active kinase conformation (type I inhibitors) might therefore have a higher risk of clinical liabilities due to lack of selectivity against other kinases. The ATP-binding site changes shape and becomes structurally less conserved when it is in a down-regulated conformation. Kinase inhibitors that target the ATP-binding site in a down-regulated conformation (type II inhibitors) might therefore have better selectivity and specificity, and hopefully a better clinical profile. Glivec/Gleevec is such a type II kinase inhibitor. While not being perfectly selective, it targets the ATP site of Bcr-Abl in its down-regulated conformation.[4,10] The best selectivity profile might be possible for inhibitors that bind outside the ATP site, at the substrate site or an allosteric binding site. These sites are not generally conserved, and high selectivity against other kinases can hopefully be achieved.[9,11–13]

Most known kinase inhibitors are type I or II inhibitors.[8,14] This is probably due to the fact that most kinase inhibitor screens are performed by using biochemical functional assays with purified and activated recombinant kinase. Allosteric kinase inhibitors are not identified by these assay types if they do not inhibit kinase catalysis per se, although they might inhibit kinase activation or signal transduction. Allosteric kinase

[a] *Dr. W. Jahnke, Dr. M. J. J. Blommers, Dr. C. Fernández, C. Zwingelstein, Dr. R. Amstutz*
Novartis Institutes for Biomedical Research, Discovery Technologies
WSJ-88.904, 4002 Basel (Switzerland)
Fax: (+41) 61-324-2686
E-mail: wolfgang.jahnke@novartis.com

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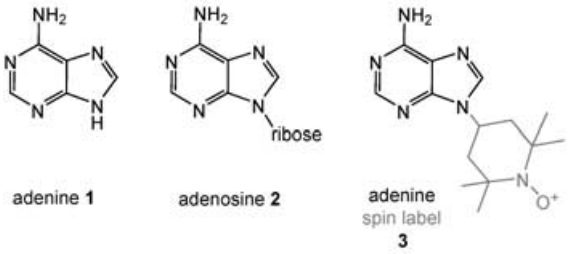
inhibitors can be identified in cellular assays or in biophysical binding assays, for example.

NMR spectroscopy provides a robust biophysical binding assay with high sensitivity for weak binding interactions. NMR also offers the possibility to characterize a protein–ligand interaction by structural methods or by competition experiments. Allosteric kinase ligands can, in principle, be identified by using established NMR screening methods in the presence of high concentrations of adenine or an adenine derivative (AMP, ATP, or the nonhydrolyzable ATP analogue, AMPPNP), or in the absence of an adenine derivative but followed by competition experiments with an adenine derivative. Those hits that are not competitive with the adenine derivative are expected to bind outside of the ATP-binding site. Unfortunately, the interpretation of these competition experiments is complicated, since ATP binding can lead to a conformational change in the allosteric binding site, so that the affinity of an allosteric ligand can be modulated by ATP binding, which can easily be misinterpreted as competitive binding. In this communication, we describe a novel method to identify kinase inhibitors that bind outside the ATP-binding site, followed by a description of our protocol for NMR-based optimization of these inhibitors.

Our approach to the detection of allosteric kinase inhibitors involves a spin-labeled adenine analogue, such as **3**. Spin



labels, like the NO radical, have a free electron and are paramagnetic. They exert drastic and long-ranging paramagnetic relaxation enhancement effects on any nuclear spin within a distance of 15–20 Å.[15–17] This feature has been widely used for the structural characterization of proteins, and we have demonstrated its use for the detection and characterization of protein–ligand interactions.[18,19] Spin labeling of the protein as in the SLAPSTIC experiment[19] allows for efficient identification of any protein ligand, while spin labeling of a given ligand permits the identification of a second ligand that binds simultaneously and in the vicinity of the first ligand.[18] The identification of such a second-site ligand is key for the linked-fragment strategy of fragment-based ligand design.[20]

The use of spin-labeled ligands can be further extended to the identification of allosteric inhibitors when the binding site of a known ligand is close to an allosteric site. This applies to kinases. If an adenine derivative is spin-labeled, another kinase ligand that binds to a second site will feel the paramagnetic relaxation enhancement effects if and only if it is bound simultaneously and within 15–20 Å of the spin-labeled adenine derivative (Figure 1). If the other ligand binds to the ATP-binding site, the same as the spin-labeled adenine derivative, the ligands will never be bound at the same time, and no paramagnetic relaxation enhancement effects will be conferred on the second ligand. This allows unambiguous identification of ligands that bind outside the ATP-binding site, including at an allosteric binding site. In addition, when working with down-regulated kinases, ligands that bind within the extended ATP-binding site can be identified.

Figure 2 shows a typical profile of a kinase ligand that binds outside the ATP-binding site. T1ρ relaxation spectra are shown with a short (10 ms) relaxation period in black and a long (200 ms) relaxation period in gray. A weaker signal in the 200 ms spectrum corresponds to faster relaxation, which can be indicative of protein binding or of paramagnetic relaxation enhancement. Figure 2A shows spectra of the free compound, which relaxes slowly as expected for a small molecule. Upon addition of kinase (Figure 2B), faster relaxation is observed; this indicates binding of the compound to the kinase. Relaxation is increased even further when the spin-labeled adenine analogue **3** is added. This is the crucial experiment and shows that the compound binds to a binding site distinct from but within 15–20 Å of the ATP-binding site. The degree of paramagnetic relaxation enhancement depends on the concentrations of protein (here 5 μM), test compound (200 μM), and spin label (50 μM), on the affinities of test compounds and spin label, and on the distance between test compound and spin label. Figure 2D shows a control experiment in which the spin label has been reduced by addition of ascorbic acid. Relaxation is slowed down again; this indicates that the enhanced relaxation after addition of the spin label is, in fact, due to paramagnetic relaxation enhancement. In a separate control experiment, compound and spin label **3** were added in the same concentrations as for Figure 2C, but without kinase. The resulting spectrum was indistinguishable from the spectra shown in Figure 2A; this indicates that there is no direct interaction between spin label and inhibitor, and that only the kinase brings those molecules into spatial proximity.

Care has to be taken to ensure that the spin-labeled adenine analogue binds exclusively to the ATP-binding site. If it additionally bound to another binding site, a test compound binding to the ATP site would yield an identical profile to that shown in Figure 2, and would therefore be misinterpreted as allosteric ligand. Several control experiments are suitable to test for nonspecific binding of the spin-labeled adenine analogue. After reduction of the spin label (to make the signals of the spin label visible), the spin label should be completely displaced by excess ATP or AMPPNP. In addition, for experiments in which an adenine derivative is added as "test compound", no paramagnetic relaxation enhancement should be observed for the adenine derivative in the presence of spin-labeled adenine. In our experiments, some kinases were found to bind the spin-labeled adenine **3** nonspecifically. For these kinases, more hydrophilic spin-labeled adenine analogues might reduce the amount of nonspecific binding. Work along these lines is in progress. Moreover, paramagnetic metals cause drastic relaxation effects on neighboring nuclear spins. Kinases bind Mg^{2+}/ATP, and Mg^{2+} can be replaced by paramagnetic Mn^{2+}. Kinases

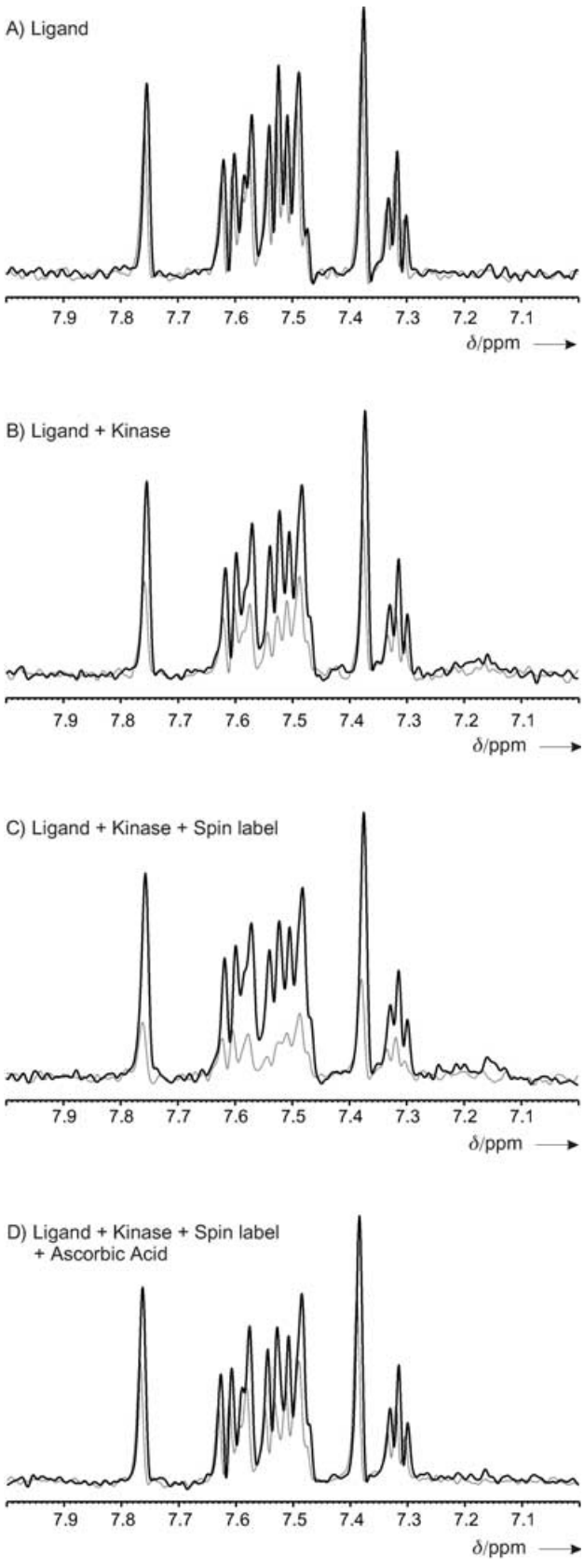


Figure 2. NMR profile of an allosteric kinase inhibitor. T1ρ relaxation spectra are shown with relaxation periods of 10 ms (black) and 200 ms (gray). The concentrations used were 5 μM kinase, 200 μM inhibitor, and 50 μM **3.** The spectra were recorded at 296 K on a Bruker DRX600 NMR spectrometer with 256 scans each.

complexed to Mn^{2+}/ATP can also be used to screen for novel ligands.[21] However, care must be taken since Mn^{2+} binds non-specifically to several protein sites.

The affinities of fragments that are allosteric kinase inhibitors are generally very weak, and methods to improve their affinity are an integral part of fragment-based ligand design. As discussed above, allosteric kinase inhibitors cannot always be detected in a simple enzymatic assay with purified and activated kinase, so that IC_{50} determination for the optimization of allosteric inhibitors cannot always be performed in biochemical assays. Therefore an NMR-based strategy is described in the following. Compounds can be conveniently optimized with respect to potency by using competition-based formats, such as NMR reporter screening.[22–24] Reporter screening measures the ability of test compounds to displace a "reporter ligand" bound to the protein target. It allows the determination of K_D for a test compound relative to the K_D of the reporter ligand. If the K_D of the reporter ligand has been determined by using other methods, such as isothermal titration calorimetry (ITC) or chemical shift mapping, absolute K_D values can be deduced by reporter screening. For practical purposes, relative K_D values are often sufficient. The degree of reporter-ligand displacement as function of test-compound affinity (and concentration) depends on a variety of parameters, and can be solved numerically or analytically.[25] It can also be experimentally determined in a straightforward way by using T1ρ relaxation experiments. As can be confirmed by numerical simulations, the T1ρ relaxation rate of a reporter ligand at double concentration is identical to its T1ρ relaxation rate at the original concentration, if a test compound with equal affinity is added at equal concentration. This procedure allows experimental calibration of reporter-ligand displacement and is useful for quickly identifying compounds with higher affinity than the reporter ligand.

Quantization of reporter screening generally works best for test compounds with K_D values within one order of magnitude of that of the reporter ligand. If the potency of a test compound is higher by more than one order of magnitude, the reporter ligand is almost completely displaced, and quantization becomes imprecise. In order to alleviate this limitation, the current reporter ligand can be replaced by a more potent one as soon as a more potent compound has been identified, for example by screening compounds selected from similarity searches or chemical-optimization efforts (Figure 3). By performing this "reporter hopping", one can gradually increase the potency of test compounds, thereby allowing precise quantization of the binding affinity. As an added benefit, protein demands become less as the potency of compounds increases, since less protein is generally needed to detect higher-affinity compounds.

The described strategies for identification and optimization of allosteric kinase inhibitors have the potential to facilitate the discovery of non-ATP-competitive kinase inhibitors that might exhibit better selectivity profiles than ATP-competitive inhibitors.

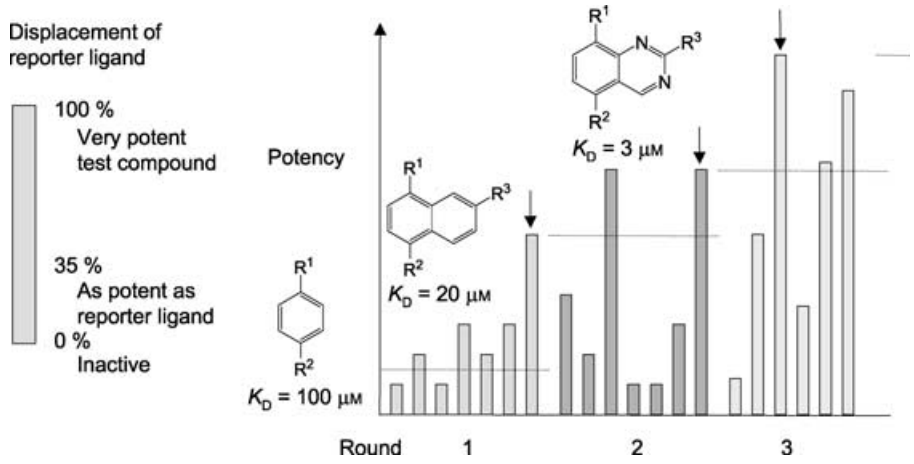


Figure 3. Principle of "reporter hopping". A weakly binding ligand (K_D = 100 μM), typically from a fragment screen, is taken as reporter ligand, and a follow-up library of compounds, selected by similarity search or from a chemical optimization series, is screened for their ability to displace the reporter ligand. The degree to which a test compound with equal or higher binding affinity displaces the reporter ligand can be calibrated beforehand (see text). If better test compounds are identified in round 1, the best of these becomes the new reporter ligand (K_D = 20 μM). A new displacement calibration is undertaken, and a second follow-up library is screened in round 2 to identify ligands that are better than the 20 μM reporter ligand, and so on. This iterative screening against improved reporter ligands allows a rapid assessment of compound potency improvement.

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