doi:10.2533/chimia.2015.421 Chimia 69 (2015) 421–424

Contributions of Biomolecular NMR to Allosteric Drug Discovery

Lukasz Skora and Wolfgang Jahnke§*

§KGF-SCS Industrial Investigator Award 2014

Abstract: Drug discovery is a complex process, and a variety of technologies contribute to its success. Biophysical methods have gained widespread attention within the last decade, and in particular NMR spectroscopy as the most versatile biophysical method has seen numerous applications and significant impact to drug discovery. Here we summarize the potential of NMR to support drug discovery, and highlight a number of recent applications.

Keywords: Allosteric drug discovery · Biomolecular NMR

Wolfgang Jahnke *is a director for Structural Biophysics in the Center for Proteomic Chemistry at the Novartis Institutes for Biomedical Research in Basel. He studied chemistry at the Universität Tübingen and the University of Arizona, and received his PhD after work with Prof. Horst Kessler at the Technische Universität München and with Dr. Peter Wright at the Scripps Research Institute. His main interest is in supporting drug discovery projects by the application of NMR, other biophysical methods, and fragment-based approaches.*

*Correspondence: Dr. W. Jahnke
Novartis Institutes for Biomedical Research
Novartis Campus
CH-4002 Basel
Tel.: +41 79 845 3230
E-mail: wolfgang.jahnke@novartis.com

Introduction

Several biophysical methods are now available to support target-based drug discovery, and have proven useful to aid in the discovery and optimization of hits and lead compounds up to clinical candidates.[1] Surface plasmon resonance (SPR) has been widely applied to measure affinity and binding kinetics of ligands to macromolecular targets. Differential scanning calorimetry (DSF) is a popular approach to measure the thermal stabilization of proteins by ligands and is often used as an assay to measure ligand binding. Isothermal titration calorimetry (ITC) can be used for a detailed measurement of thermodynamic parameters upon ligand binding, and emerging methods such as microscale thermophoresis (MST) have the potential to complement and enhance existing methods to quantify protein-ligand association with even higher throughput and lower protein requirements.

Nuclear magnetic resonance (NMR) spectroscopy is the most versatile and robust of all biophysical methods applied to drug discovery, and principles of NMR in drug discovery, especially using fragment-based approaches, have been the subject of multiple reviews.[2] Its versatility allows tailor-made solutions to particular problems and its robustness makes it ideally suited for the discovery of allosteric inhibitors of enzymes and protein–protein interactions (PPIs). Whereas other biophysical methods often rely on competitive binding experiments and are thus better suited for drug discovery at orthosteric sites, or allosteric sites for which tool compounds exist, NMR can reliably detect ligand binding even in the absence of tool compounds. Both features, the versatility and robustness of NMR, have been employed in the drug discovery examples below.

In the following, we will review recent examples of drug discovery projects where NMR was able to provide support by binding data and/or structural information. All examples describe the discovery of allosteric inhibitors. Such kinds of inhibitors are typically difficult to identify because they are not always picked up in standard assays, and biophysical techniques are not always straightforward if there is no precedence, *i.e.* if no tool compounds exist to validate assays and provide molecules for competition experiments. However, allosteric inhibitors often have the preferred mode of action because they potentially have better selectivity profiles and can be combined with orthosteric inhibitors to provide greater efficacy.

Discovery of ABL001, an Allosteric Bcr-Abl Inhibitor

Chronic myelogenous leukemia is caused by a reciprocal chromosomal translocation by which part of chromosome 9, carrying the *ABL1* gene, is fused to part of chromosome 22, carrying the *BCR* gene. The resulting 'Philadelphia chromosome' contains the fusion protein Bcr-Abl, in which Abelson (Abl) kinase is constitutively activated, leading to uncontrolled proliferation, lack of differentiation, and reduced apoptosis of haematopoietic cells.[3] The activity of c-Abl is tightly regulated by an autoinhibitory mechanism that involves binding of myristate, which is covalently linked to the N-terminus of c-Abl, to a pocket in the C-terminal lobe of the catalytic domain (Fig. 1).[4] The so-formed assembled inactive state lacks the flexibility and conformational freedom neces-

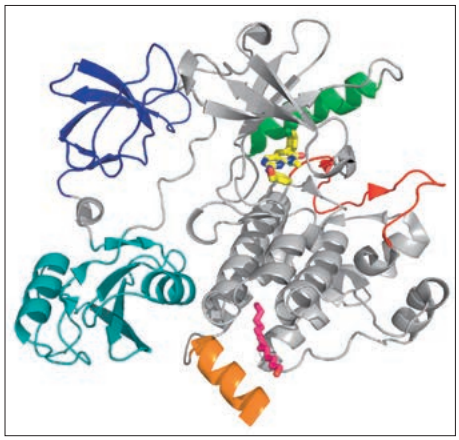

Fig. 1. Abl kinase in the assembled inactive state (pdb: 2FO0).[4b] The SH3 (blue) and SH2 (cyan) domains dock against the kinase domain, caused by binding of N-terminally attached myristate (magenta sticks) into the myristate pocket. The ATP-pocket is filled with PD166326 (yellow sticks). Within the kinase domain, helix I is colored orange, helix C is colored green, and the activation loop is colored red.

sary for catalytic function, and needs to be disrupted for the kinase to become active. The fusion protein, Bcr-Abl, lacks the covalently bound myristate, and correspondingly lacks the regulatory element required for autoinhibition, thus promoting its constitutive activation.

A chemical modality that restores autoinhibition by binding to the myristate pocket and inducing the assembled inactive state could have therapeutic potential as a novel Bcr-Abl inhibitor. Highly potent Bcr-Abl inhibitors exist, such as imatinib, nilotinib or dasatinib, and are in clinical practice. They all bind to the ATP-binding site of Abl and are clinically very efficacious. However, some patients are intolerant or become resistant to these therapies, so that there is still a medical need for new inhibitors, preferably with a novel mode of action.

Inspired by the serendipitous discovery of GNF-5,[5] we started a fragment-based screening campaign to identify novel allosteric inhibitors of Bcr-Abl that bind to the myristate pocket. We started by screening our fragment library against the imatinib-bound form of Abl kinase, so that all identified hits would automatically be allosteric, and used NMR spectroscopy because of its robustness. Much to our satisfaction, the hit rate was quite high (6%), and some hits bound with an extraordinary ligand efficiency (0.6). However, none of the hits was active in any functional assay, leading us to contemplate the essential features of allosteric Abl inhibition. We realized that a conformational change in the C-terminal helix I was required for inhibition, and any ligand that acts as an inhibitor has to induce this conformational change, referred to as 'bending' of the helix. Crystallographic studies, medicinal chemistry and the development and analysis of an NMR-based conformational assay[6] (Fig. 2) allowed us to modify our fragment hits and follow-up compounds, and convert them into functional inhibitors. This finally resulted in the development of ABL001, a potent and selective allosteric inhibitor of Bcr-Abl, which is currently in clinical development for the treatment of CML.[7]

In parallel to these drug discovery efforts, and in a collaboration with the Biocenter at the University of Basel, we characterized the behavior of Abl kinase complexed with several ATP-site and allosteric inhibitors. Surprisingly, a hitherto undescribed novel conformation of Abl kinase was discovered, termed the open inhibited state.[8] This state, which is adopted after binding of ATP-site inhibitors such as imatinib, nilotinib or dasatinib, is characterized by the release of the SH2 and SH3 domains from the kinase (SH1) domain. This conformation can be thought of as an intermediate conformation between the assembled inactive state (Fig. 1) and the fully active state, in which the SH2 domain is positioned on top of the N-lobe of the kinase domain.[9] Hence, there are three levels that characterize the activation state of Abl kinase: 1) The accessability of the active site for ATP binding; 2) the position of the activation loop in a DFG-in ('active') or DFG-out ('inactive') conformation; 3) formation or release of the assembled inactive state. While ATP-site inhibitors block access of ATP to the active site and may bind in DFG-in or DFG-out conformations, we now know that they actually disrupt the assembled inactive state. Allosteric inhibitors, on the other hand, stabilize the assembled inactive state but do not block access to the ATP-site. A combination of ATP-site and allosteric inhibitors,[10] however, can block the active site, induce the DFG-out ('inactive') conformation of the activation loop, and stabilize the assembled inactive state, thus balancing every possible knob towards Abl inhibition.

Discovery of Allosteric Inhibitors of Farnesyl Pyrophosphate Synthase (FPPS), the Bisphosphonate Target

Bisphosphonates, such as zoledronate, risedronate, or alendronate, are widely used drugs against bone diseases such as osteoporosis, bone metastases and Paget's disease. Their excellent safety profile comes from their peculiar pharmacokinetic properties, which are dominated by rapid and strong binding to bone mineral, resulting in low blood levels shortly after administration. Also, bisphosphonates have low cellular permeability and do not rapidly enter non-endocytic cells. They are selectively taken up by osteoclasts – their desired target cells – through endocytosis, while being bound to bone mineral.

Within the past decade, several additional therapeutic effects for bisphosphonates have been described in animal models or in clinical studies. The best studied and documented 'beneficial side effect' of zoledronate (as opposed to effects on bone) is its direct anti-tumor effect observed in breast cancer[11] and multiple myeloma patients.[12] In essence, the breast cancer study suggests that zoledronate, when added to endocrine therapy, significantly reduces the risk of relapse after surgery. Other beneficial effects of bisphosphonates, although not yet proven clinically, include anti-parasitic activity in Chagas disease and Leishmanias,[13] increased longevity in a mouse model of progeria,[14] and reduced risk of atherosclerosis.[15] Although the exact mechanism of action has not been elucidated for all of these beneficial effects, it is likely that they all occur *via* inhibition of farnesyl pyrophosphate synthase (FPPS),

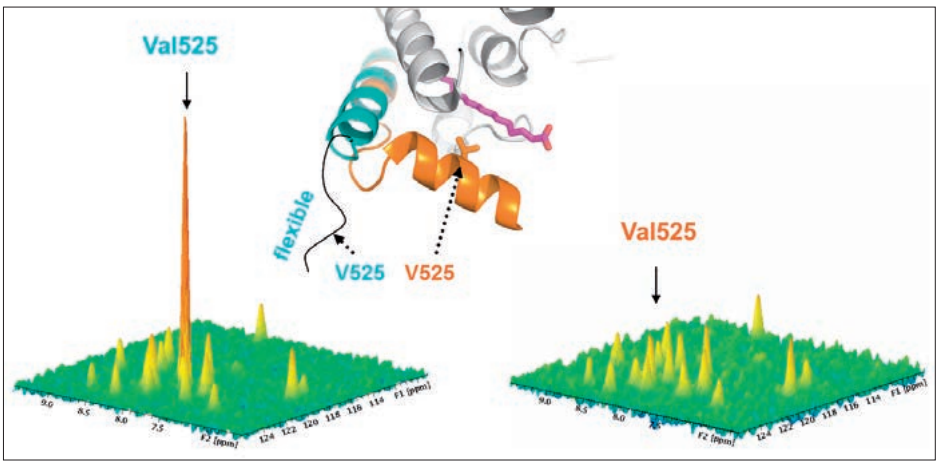

Fig. 2. NMR-based conformational assay to detect the conformation of helix I in the extended (blue, left) or bent state (orange, right).[6]

an essential enzyme in the mevalonate pathway that provides the lipids required for post-translational prenylation of G proteins, as well as cholesterol, dolichol and other essential metabolites.

The above-mentioned peculiar pharmacokinetic profile of bisphosphonates suggests that they may not be the ideal drugs for the treatment of non-bone diseases. However, bisphosphonates are substrate mimetics of dimethylallyl pyrophosphate, and any attempt to modify the bisphosphonate moiety has proven unsuccessful.

In order to discover non-bisphosphonate inhibitors of FPPS, we performed a fragment-based screen (on the apo protein) using NMR spectroscopy. We were quite surprised about the high hit rate, since none of the fragments in our library was close to bisphosphonates. Crystallographic analyses revelead that all fragments bound to a newly discovered allosteric site, situated in the vicinity but not overlapping with the active site (Fig. 3). The significance of this discovery stems from the fact that this allosteric pocket can be filled by non-bisphosphonate compounds, which is not readily possible for the active site.

Using crystallography, medicinal chemistry and structure-based design, the fragment hits could rapidly be improved and developed into nanomolar non-bisphosphonate inihbitors of FPPS, which are completely devoid of any bone affinity.[16] This initial lead series has recently been complemented by two additional chemotypes with even higher potency. These compounds can now be evaluated for use in non-bone diseases. In addition, the chemotypes have been modified with a bone-affinity tag to confer upon them weak and adjustable bone affinity, while potentially retaining oral bioavailability. An NMR-based bone binding assay was developed for this purpose and was used to quantify the affinity to bone mineral.[17] This allows the manipulation of bone affinity as an additional parameter for the treatment of bone diseases.

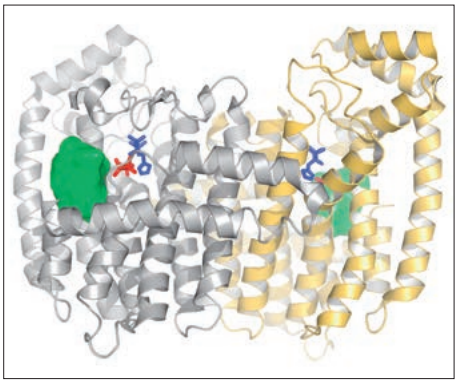

Fig. 3. Structure of farnesyl pyrophosphate synthase (FPPS). The picture shows the FPPS homodimer and indicates the position of the substrate binding sites (blue and red sticks) and the allosteric pocket (green cloud).[16]

Discovery of Allosteric Pak1 Inhibitors

PAKs (or p21-activated kinases) are a family of serine/threonine protein kinases that are effectors of Rac/Cdc42 GTPases. They play an important role in cell proliferation, survival, motility and angiogenesis, and some PAK isoforms (particularly Pak1 and Pak4) are considered as targets for oncology.[18] Since kinase selectivity is generally an issue not only for the therapeutic use of inhibitors, but also for their use as tool compounds to elucidate the roles of individual Pak isoforms, we were interested in the discovery of allosteric Pak1 inhibitors with selectivity against other kinases and against other Pak isoforms.

For this project, fragment screening was part of an integrated lead finding strategy including high-throughput screening and *in silico* approaches. The Novartis fragment library[19] was screened by NMR against Pak1, and multiple hits were obtained, among them typical kinase hinge binders, and (hopefully) allosteric ligands. In order to distinguish the (truly interesting) allosteric inhibitors from the (less interesting) hinge binders, all hits were initially reviewed for lack of a hinge-binding motif, and for non-planar shape. Similarity searches and data mining from a previous high-throughput screen gave candidates for allosteric Pak1 ligands, but no crystal structure could initially be obtained for a definite proof. Only after a rigorous search for analogs that were highly soluble, had low crystallinity, and lacked non-specific binding even at high concentrations could a crystal structure be obtained. This crystal structure showed the compound binding to the allosteric backpocket of the kinase, underneath the C helix. The structure also explained the competitive behavior of these allosteric compounds with ATP-site ligands: Even though the compound binds at a different site, the position of the activation loop allows for binding of one compound only in the absence of the other, *i.e.* binding of ATP-site ligands and allosteric ligands is mutually exclusive. These allosteric ligands were further optimized into potent and highly selective Pak1 inhibitors, which could be used as tool compounds to investigate the role of Pak1 in tumor maintenance.[20]

Conclusions

The three examples above show that allosteric ligands can be extremely selective, and have dramatically different pharmacokinetic properties compared to orthosteric ligands. NMR is an ideal method for the discovery and characterization of allosteric ligands, due to its robustness and sensitivity for weak interactions. Furthermore, NMR is an extremely versatile biophysical method which can be used to provide tailor-made solutions, as shown here by the development of a conformational assay for Abl kinase, or the development of a bone-binding assay for FPPS inhibitors.

Clearly, NMR is only one technology out of the entire toolbox for drug discovery. It is important to choose the method that is best suited to solve the particular problem. In general, multiple technologies are needed for any given drug discovery project, and tight integration of NMR with other methods, most notably X-ray crystallography, but also SPR, DSF and other biophysical methods, can generate best success.

Acknowledgements

We wish to thank our colleagues from the biomolecular NMR group in Basel (A. Blechschmidt, M. Blommers, S. Chau, P. Erbel, C. Fernandez, A. Gossert, C. Henry, U. Hommel, S. Rüdisser, A. Wiget) for excellent collaboration and a great team. All projects were carried out in collaboration with the respective project team, and we wish to thank the team members for their support, most notably A. Marzinzik, S. Jacob, A. Wylie, J. Schoepfer, G. Vanasse, J.-M. Rondeau, M. Lindvall and A. Karpov.

Received: June 5, 2015

[1] 'Biophysical Approaches Determining Ligand Binding to Biomolecular Targets: Detection, Measurement and Modelling', Eds. A. Podjarny, A. P. Dejaegere, B. Kieffer, The Royal Society of Chemistry, Cambridge, **2011**.

[2] a) M. Pellecchia, I. Bertini, D. Cowburn, C. Dalvit, E. Giralt, W. Jahnke, T. L. James, S. W. Homans, H. Kessler, C. Luchinat, B. Meyer, H. Oschkinat, J. Peng, H. Schwalbe, G. Siegal, *Nat. Rev. Drug Discov.* **2008**, *7*, 738; b) C. Farenc, G. Siegal, in 'Recent Developments in Biomolecular NMR', Eds. M. Clore, J. Potts, The Royal Society of Chemistry, **2012**, p. 254; c) T. Maurer, *Methods Enzymol.* **2011**, *493*, 469; d) H. Widmer, W. Jahnke, *Cell. Mol. Life Sci.* **2004**, *61*, 580; e) M. J. Harner, A. O. Frank, S. W. Fesik, *J. Biomol. NMR.* **2013**, *56*, 65.

[3] R. Ren, *Nat. Rev. Cancer* **2005**, *5*, 172.

[4] a) O. Hantschel, B. Nagar, S. Guettler, J. Kretzschmar, K. Dorey, J. Kuriyan, G. Superti-Furga, *Cell* **2003**, *112*, 845; b) B. Nagar, O. Hantschel, M. A. Young, K. Scheffzek, D. Veach, W. Bornmann, B. Clarkson, G. Superti-Furga, J. Kuriyan, *Cell* **2003**, *112*, 859.

[5] F. J. Adrian, Q. Ding, T. Sim, A. Velentza, C. Sloan, Y. Liu, G. Zhang, W. Hur, S. Ding, P. Manley, J. Mestan, D. Fabbro, N. S. Gray, *Nat. Chem. Biol.* **2006**, *2*, 95.

[6] W. Jahnke, R. M. Grotzfeld, X. Pelle, A. Strauss, G. Fendrich, S. W. Cowan-Jacob, S. Cotesta, D. Fabbro, P. Furet, J. Mestan, A. L. Marzinzik, *J. Am. Chem. Soc.* **2010**, *132*, 7043.

[7] *https://www.nibr.com/stories/medicines/cancer-cocktails-abl001-%E2%80%93-hitting-one-target-twice*, accessed May 30, **2015**.

[8] L. Skora, J. Mestan, D. Fabbro, W. Jahnke, S. Grzesiek, *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, E4437.
[9] B. Nagar, O. Hantschel, M. Seeliger, J. M. Davies, W. I. Weis, G. Superti-Furga, J. Kuriyan, *Mol. Cell* **2006**, *21*, 787.
[10] J. Zhang, F. J. Adrian, W. Jahnke, S. W. Cowan-Jacob, A. G. Li, R. E. Iacob, T. Sim, J. Powers, C. Dierks, F. Sun, G. R. Guo, Q. Ding, B. Okram, Y. Choi, A. Wojciechowski, X. Deng, G. Liu, G. Fendrich, A. Strauss, N. Vajpai, S. Grzesiek, T. Tuntland, Y. Liu, B. Bursulaya, M. Azam, P. W. Manley, J. R. Engen, G. Q. Daley, M. Warmuth, N. S. Gray. *Nature* **2010**, *463*, 501.
[11] M. Gnant, B. Mlineritsch, H. Stoeger, G. Luschin-Ebengreuth, D. Heck, C. Menzel, R. Jakesz, M. Seifert, M. Hubalek, G. Pristauz, T. Bauernhofer, H. Eidtmann, W. Eiermann, G. Steger, W. Kwasny, P. Dubsky, G. Hochreiner, E. P. Forsthuber, C. Fesl, R. Greil, Austrian Breast and Colorectal Cancer Study Group, Vienna, Austria, *N. Engl. J. Med.* **2009**, *360*, 679.
[12] G. J. Morgan, F. E. Davies, W. M. Gregory, K. Cocks, S. E. Bell, A. J. Szubert, N. Navarro-Coy, M. T. Drayson, R. G. Owen, S. Feyler, A. J. Ashcroft, F. Ross, J. Byrne, H. Roddie, C. Rudin, G. Cook, G. H. Jackson, J. A. Child, National Cancer Research Institute Haematological Oncology Clinical Study Group, *Lancet* **2010**, *376*, 1989.
[13] R. Docampo, S. N. Moreno, *Curr. Drug Targets Infect. Disord.* **2001**, *1*, 51.
[14] I. Varela, S. Pereira, A. P. Ugalde, C. L. Navarro, M. F. Suarez, P. Cau, J. Cadinanos, F. G. Osorio, N. Foray, J. Cobo, F. de Carlos, N. Levy, J. M. P. Freije, C. Lopez-Otin, *Nat. Med.* **2008**, *14*, 767.
[15] R. Ylitalo, *Gen. Pharmacol.* **2000**, *35*, 287.
[16] W. Jahnke, J. M. Rondeau, S. Cotesta, A. Marzinzik, X. Pelle, M. Geiser, A. Strauss, M. Götte, F. Bitsch, R. Hemmig, C. Henry, S. Lehmann, J. F. Glickman, T. P. Roddy, S. J. Stout, J. R. Green, *Nat. Chem. Biol.* **2010**, *6*, 660.
[17] W. Jahnke, C. Henry, *ChemMedChem*. **2010**, *5*, 770.
[18] D. Z. Ye, J. Field, *Cell. Logist.* **2012**, 105.
[19] P. S. Kutchukian, A. M. Wassermann, M. K. Lindvall, S. K. Wright, J. Ottl, J. Jacob, C. Scheufler, A. Marzinzik, N. Brooijmans, M. Glick, *J. Biomol. Screen.* **2015**, *5*, 588.
[20] A. S. Karpov, P. Amiri, C. Bellamacina, M.-H. Bellance, W. Breitenstein, D. Daniel, R. Denay, D. Fabbro, C. Fernandez, I. Galuba, S. Guerro-Lagasse, S. Gutmann, L. Hinh, W. Jahnke, J. Klopp, A. Lai, M. K. Lindvall, S. Ma, H. Möbitz, S. Pecchi, G. Rummel, K. Shoemaker, J. Trappe, C. Voliva, S. W. Cowan-Jacob, A. L. Marzinzik, *ACS Med. Chem. Lett.* **2015**, Epub May 22, DOI 10.1021/acsmedchemlett.5b00102.