Methods and Principles in Medicinal Chemistry

Edited by Bert Klebl, Gerhard Müller,
and Michael Hamacher

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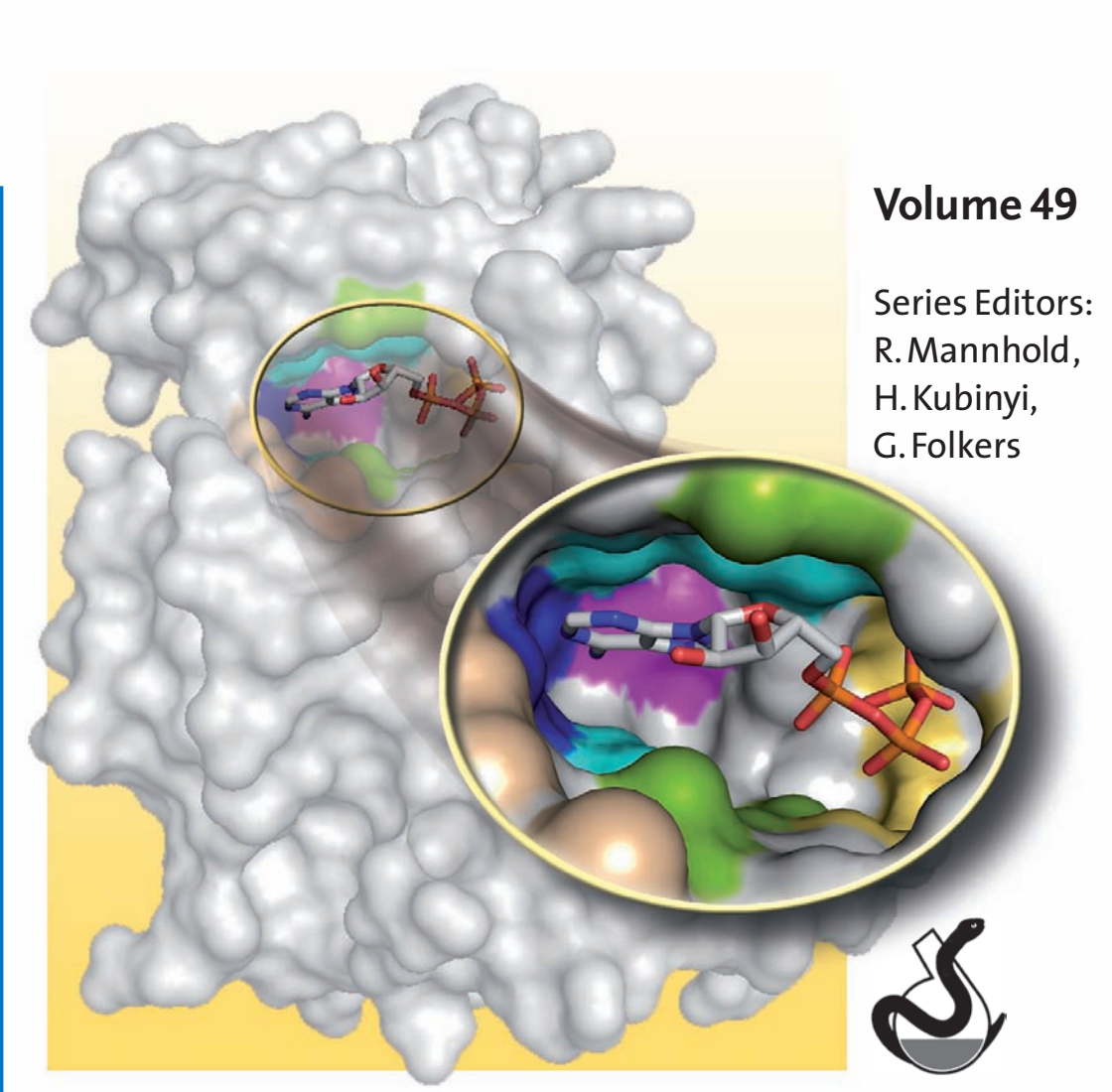
Protein Kinases as Drug Targets



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Bert Klebl, Gerhard Müller,
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Series Editors

Prof. Dr. Raimund Mannhold
Molecular Drug Research Group
Heinrich-Heine-Universität
Universitätsstrasse 1
40225 Düsseldorf
Germany
mannhold@uni-duesseldorf.de

Prof. Dr. Hugo Kubinyi
Donnersbergstrasse 9
67256 Weisenheim am Sand
Germany
kubinyi@t-online.de

Prof. Dr. Gerd Folkers
Collegium Helveticum
STW/ETH Zurich
8092 Zurich
Switzerland
folkers@collegium.ethz.ch

Volume Editors

Dr. Bert Klebl
Lead Discovery Center GmbH
Emil-Figge-Straße 76 a
44227 Dortmund
Germany

Dr. Gerhard Müller
Proteros Fragements GmbH
Am Klopferspitz 19
82152 Planegg
Germany

Dr. Michael Hamacher
Lead Discovery Center GmbH
Emil-Figge-Str. 76 a
44227 Dortmund
Germany

Cover Description
ATP binding site of the Cyclin-dependent protein kinase 7 (CDK7), a member of the CDK family involved in the regulation of the cell cycle and transcription. The kinase active site is divided in sub-sites according to its interactions, varying between individual enzymes and allowing the indiviual design of selective inhibitors. (Photo courtesy C. McInnes)

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Contents

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List of Contributors

Tom Alber
University of California
Department of Molecular and Cell Biology
374B Stanley Hall #3220
Berkeley, CA 94720-3220
USA

Yossef Av-Gay
University of British Columbia
Department of Medicine
Division of Infectious Diseases
Vancouver, British Columbia
Canada V5Z 3J5

Alexander C. Backes
Sandoz GmbH
Sandoz Development Center
Biochemiestrasse 10
6336 Langkampfen
Austria

Matthias Baumann
Lead Discovery Center GmbH
Emil-Figge-Str 76a
44227 Dortmund
Germany

Axel Choidas
Lead-Discovery Center GmbH
Emil-Figge-Straße 76a
44227 Dortmund
Germany

Jan Eickhoff
Lead-Discovery Center GmbH
Emil-Figge-Straße 76a
44227 Dortmund
Germany

Doris Hafenbradl
BioFocus AG
Gewerbestrasse 16
4123 Allschwil
Switzerland

Nicola Heron
Devices for Dignity
Sheffield Teaching Hospitals NMS Foundation Trust
Royal Hallamshire Hospital
Glossop Road Sheffield, S10 2YF
UK

György Kéri
Vichem Chemie Research Ltd.
Herman Ottó u. 15
1022 Budapest
Hungary

Protein Kinases as Drug Targets. Edited by B. Klebl, G. Müller, and M. Hamacher

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and

Semmelweis University
Hungarian Academy of Sciences
Pathobiochemical Research Group
Tüzoltó u. 37-47
1094 Budapest
Hungary

George Kontopidis
University of Thessaly
Veterinary School
Department of Biochemistry
43100 Karditsa
Greece

Stefan Laufer
Eberhard-Karls-Universität Tübingen
Pharmazeutisches Institut
Auf der Morgenstelle 8
72076 Tübingen
Germany

Campbell McInnes
South Carolina College of Pharmacy
715 Sumter St.
Columbia, SC 29208
USA

Mokdad Mezna
Beatson Institute for Cancer Research
Translational Research
Garscube Estateswitchback Road
Glasgow G61 1BD
UK

Gerhard Müller
Proteros Fragments GmbH
Fraunhoferstr. 20
82152 Martinsried
Germany

Gábor Németh
Vichem Chemie Research Ltd.
Herman Ottó u. 15
1022 Budapest
Hungary

Lars Neumann
Proteros Biostructures
Am Klopferspitz 19
82152 Martinsried
Germany

László Őrfi
Vichem Chemie Research Ltd.
Herman Ottó u. 15
1022 Budapest
Hungary

and

Semmelweis University
Department of Pharmaceutical Chemistry
Högyes Endre u. 9
1092 Budapest
Hungary

Matthias Rabiller
Chemical Genomics Centre of the Max Planck Society
Otto-Hahn-Str. 15
44227 Dortmund
Germany

Daniel Rauh
Chemical Genomics Centre of the Max Planck Society
Otto-Hahn-Str. 15
44227 Dortmund
Germany

Luis M. Schang
University of Alberta
Department of Biochemistry
327 Heritage Medical Research Center
Edmonton, Alberta
Canada, T6G 2S2

Peter C. Sennhenn
Proteros Fragments GmbH
Fraunhoferstr. 20
82152 Martinsried
Germany

Jeffrey R. Simard
Chemical Genomics Centre of the
Max Planck Society
Otto-Hahn-Str. 15
44227 Dortmund
Germany

Preface

Protein kinases are a huge group of evolutionary and structurally related enzymes, which by phosphorylation of certain amino acids, in first-line serine/threonine and tyrosine, activate a multitude of proteins. In this manner, they mediate signal transduction in cell growth and differentiation. The therapeutic potential of kinase inhibitors results from the crucial role kinases (as well as some kinase mutants and hybrids resulting from chromosomal translocation) play in tumor progression and in several other diseases. With a group size of more than 500 individual members, the "kinome," that is, the sum of all kinase genes, constitutes about 2% of the human genome. Since the isolation of the first Ser/Thr-specific kinase in the muscle in 1959, it took another 20 years until tyrosine protein kinases were discovered and another 20 years before the first 3D structure of a kinase was determined. Starting with the 3D structure of protein kinase A in 1991, many more structures were elucidated in the meantime, in their active and inactive forms, without and with ligands other than ATP. These structures show not only the close structural relationship between all kinases but also the high complexity of their allosteric regulation. Today, the term "protein kinase" retrieves almost 2000 entries from the Protein Data Bank of 3D structures; most of these structures are protein–ligand complexes with about 1000 different ligands. All kinases show a highly conserved binding site for ATP, and for this reason they were for long time considered nondruggable targets. This view was supported by the fact that the natural product staurosporine inhibits a huge number of kinases in a nonspecific manner. Still today, staurosporine is the most promiscuous kinase inhibitor, despite its large size. However, with increase in structural knowledge, additional pockets were discovered in direct vicinity of the binding motif of the adenine part of ATP (the "hinge region"). Step by step, these pockets were explored and kinase inhibitors of higher specificity emerged. Finally, the optimization of a PKC inhibitor to the bcr/abl tyrosine kinase inhibitor imatinib (Gleevec®, Novartis) marked a breakthrough in specific tumor therapy. Although initially designed for the treatment of chronic myelogenous leukemia, the drug turned out to be beneficial also for the treatment of gastrointestinal stromal tumors (GISTs). Several other kinase inhibitors followed, with significantly different specificity profiles. Even nonspecific inhibitors, such as sunitinib (Sutent®, Pfizer), are

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valuable anticancer drugs, in this case for the therapy of advanced kidney cancer and as the second-line treatment of GIST, in cases where Gleevec® fails. Due to the multitude of tumor forms, resulting from various mechanisms, research on kinase inhibitors is now one of the hottest topics in pharmaceutical industry. Resistance to some kinase inhibitors forces the industry to also search for analogues with a broader spectrum of inhibitory activity. As of today, nine small-molecule kinase inhibitors for the treatment of oncological diseases have reached the market and many more are in different phases of clinical development. Even the first kinase inhibitors targeted toward nononcological applications, such as inflammatory disease states, have reached late-stage clinical development.

We are very grateful to Bert Klebl, Gerhard Müller, and Michael Hamacher who assembled a team of leading scientists for discussion of various topics of protein kinase inhibitors, including assay development, hit finding and profiling, medicinal chemistry, and application of kinase inhibitors to various therapeutic areas. We are also very grateful to all chapter authors who contributed their manuscripts on time. Of course, we appreciate the ongoing support of Frank Weinreich and Nicola Oberbeckmann-Winter, Wiley-VCH, for our book series "Methods and Principles in Medicinal Chemistry" and their valuable collaboration in this project.

September 2010

Raimund Mannhold, Düsseldorf
Hugo Kubinyi, Weisenheim am Sand
Gerd Folkers, Zürich

A Personal Foreword

Kinase inhibitors are one of the fastest emerging fields in pharmaceutical research, reigning at "No. 2" in terms of overall spending for discovery and development of pharmaceuticals, when split according to target family classes. In our own professional histories, we still witnessed the dogma in pharmaceutical industry claiming that protein kinases are considered to be nondruggable targets. This dogma was all around during the 1990s of the last millennium. Some brave individuals nevertheless pursued the idea of identifying and developing kinase inhibitors for biologically highly interesting targets, such as p38 kinases [3] and protein kinase C (PKC) isoforms [4]. Although these were groundbreaking efforts in drug discovery in those early days, p38 and PKC inhibitors have never really made it beyond the status of tool compounds for biological research and chemical biology so far. At the end, a rather serendipitous finding started the race toward the competitive generation of kinase inhibitors in oncology. The introduction of a simple methyl group into a diaminopyrimidine scaffold of a known protein kinase C inhibitor led to the generation of a relatively specific Bcr-Abl inhibitor, called imatinib or Gleevec™. The fusion protein Bcr-Abl has been known as the driving oncogene in chronic myeloid leukemias (CML) with a mutation on the Philadelphia chromosome [5], which is mediated by the elevated Abl activity of the mutant. Subsequently, imatinib has shown convincing efficacy in treating CML patients [6]. A new era started when imatinib was launched in 2001 as the first specifically designed small-molecule kinase inhibitor. The second beneficial serendipity during the generation and development of imatinib was understood only slowly. Imatinib is not just a plain and simple ATP competitor as most kinase inhibitors were designed to be. It binds to the inactive form of Bcr-Abl and keeps the kinase in its inactive conformation [7]. Today, this phenomenon is not only much better understood but also considered to be an important design element when synthesizing novel kinase inhibitors. Both serendipitous features of imatinib, inhibition of Bcr-Abl and binding to the inactive kinase, paved the way for the establishment of its clinical efficacy. However, this success gave birth to another dogma that kinase inhibitors will be useful only for developing anticancer therapies. This second dogma was based on two assumptions: (1) since 2001, imatinib has been considered to be among the most selective kinase inhibitors although it potently inhibits at least a dozen other protein kinases [8]; (2) "ATP-competitive inhibitors are never going to be highly selective, because they bind

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to the highly conserved active domain of kinases". Especially, the second point on the lack of selectivity was and still is highly speculative and led to the conclusion that nonselective kinase inhibitors cannot be used as treatment options in indication areas outside cancer because of their naturally invoked off-target mediated adverse effects. This assumption vice versa also led to the conclusion that nonselective but potent kinase inhibitors will be effective cancer killing agents. We would like to challenge these hypotheses for a number of reasons:

- Kinase inhibitor technologies quickly advanced, especially compound design technologies, facilitated by the development of molecular modeling and X-ray resolutions of a large number of kinase inhibitor cocrystals (www.pdb.org/pdb/home/home.do).
- Exploitation of inhibitor binding to the inactive form of a kinase (type II inhibitors) has become an accepted design strategy and leads to a number of advantages in the pharmacological development of kinase inhibitors.
- Monoselective ATP competitors (type I inhibitors) have been generated, despite the fact that they bind only to the active site of a kinase [9].
- A fair number of scaffolds are known to compete with ATP for binding to the kinase active site, allowing a quick screening effort to identify potential starting points for a subsequent optimization program on practically any kinase.
- Allosteric kinase inhibitors have been reported to be an option for further development [10].
- The correlation between kinase homology and parallel structure–activity relationship tends to be understood much better [11].
- Nowadays, kinase inhibitor design can be envisioned as the molecular game with Lego bricks – and it really works.

Over these past years, we have been able to generate highly specific kinase inhibitors [12]. Since kinases play a role not only in carcinogenesis but also in all sorts of physiologically relevant signaling pathways [13], we are convinced that both oncology and any other medical indication might represent an important playground for the application of selective and safe kinase inhibitors. Future will demonstrate that kinase inhibitors are going to be applied to treat chronic conditions and not only in life-threatening settings. Therefore, we have chosen contributions to this book that describe the generation and application of kinase inhibitors also outside the important field of anticancer drug discovery. Broadly specific kinase inhibitors, such as sunitinib, will not have a chance for development for indications other than cancer. Instead, monoselective kinase inhibitors or multikinase inhibitors with a narrow profile will turn out to be efficacious if the chosen target is critical enough in a particular pathophysiological process. It is more about the validation of the target(s) and the underlying target(s) rationale. In that respect, it remains to be seen if p38α turns out to be a valid target for rheumatic arthritis or to be valid only for some distinct inflammatory diseases. The odds are that p38α inhibitors will not reach the status of a general anti-inflammatory agent due to target-mediated toxicities [14]. Although all p38α inhibitor research might then be considered a lost investment, it has nonetheless contributed enormously to the general strategies in developing kinase

inhibitors, such as the directed design of type II inhibitors and the generation of highly selective kinase inhibitors, as well as their translation into pharmacologically active substances (e.g., [15]). These efforts significantly helped to pave the way for the development of highly selective future kinase inhibitors for different kinase targets without target-mediated toxicities. The world of protein kinases consists of more than, 500 individual members, the human kinome [16], therapeutically relevant parasitic kinase targets even not considered. Therefore, our prediction is that we will see many more novel drug candidates and pharmaceutical products arising from this large and important family of enzymes.

This gives hope to millions of patients suffering not only from various cancers but also from inflammatory, metabolic, and neurological disorders and infectious diseases, where a distinct kinase is out of control and must be tamed by a highly specific and potent kinase inhibitor. But what makes a good inhibitor? Which steps have to be taken for identifying a target and successfully making a drug with, if possible, no side effects? Which kinase inhibitors have been developed so far by using which design strategy? Can we already define lessons learned?

Small molecules and their apparently endless modularity and flexibility to produce all necessary structures are the perfect source for developing kinase inhibitors. Libraries of thousands to millions of compounds can be screened easily in high-throughput screens (HTS) or even *in silico*. Detected hits can be optimized step-by-step in iterative cycles toward highly potent and specific preclinical candidates and well-tolerated drugs on the market (or toward specific probes and tools in basic research). Thus, this book is dedicated to small-molecules kinase inhibitors and their various contributions to medical application.

Literature is exploding in the kinase inhibitor field, particularly when dealing with appropriate tools and design. In order to give a comprehensive overview about this special but diverse inhibitor species, this book covers the most important criteria from assay development to profiling and from medicinal chemistry-based optimization to a potential application. This book has been arranged in a logical order in various parts to highlight

- hit finding and profiling for protein kinases, describing the Dos and Don'ts while identifying and (cellular) profiling of active small-molecule kinase inhibitors.
- chemical kinomics to detect phosphorylation networks.
- medicinal chemistry, offering a detailed summary of existing kinase inhibitors, available technologies, and design principles that might be considered.
- application to therapeutic indication areas, discussing in detail success stories and unmet needs in medical application including cancer, inflammatory diseases, and infections.

Thanks to the enthusiasm and the perseverance of the authors and the publisher of this book, we finally made it. Somehow, the genesis of this small compendium on kinase inhibitor research resembles the field of small-molecule-based kinase inhibitors itself. Some brave individuals quickly wrote and delivered their contributions within a short period of time, some others took more time to develop their chapters, and finally, some opted out of the project and were replaced by others who maybe

considered newcomers to the field. This process seemed to reflect the development of the field of kinase inhibitor research over the past 15 years in nice analogy. On purpose, we have selected contributions on kinase inhibitor drug discovery from early-stage discoveries since there have been a lot of writing and comprehensive reviews on successfully launched kinase inhibitors, such as Gleevec, Iressa, Tarceva, Sorafenib, Sutent, Dasatinib, Lapatinib, and others ([1, 2] and references therein). There is also a good body of literature available on kinase inhibitors in cancer drug discovery. So, we rather focused both on the technologies for the discovery of kinase inhibitors and on the optimization of these inhibitors, and we included novel potential therapeutic applications of kinase inhibitors, especially fields outside the cancer research. Therefore, this collection of articles is quite unique, albeit highly representative when it comes to the identification and generation of novel kinase inhibitors with biological and pharmacological activity.

In the different chapters, experts in their field summarize the historical evolution, the trends, and a good part of their own experience gained while working in their respective fields. After reading the book, it will become clear how much promise small-molecule kinase inhibitors really hold, not only for the described therapeutic indications but also beyond, when obeying basic, intrinsic rules.

We are convinced that small-molecule kinase inhibitors will become ever more important in the years to come and are going to celebrate new success stories for research and patients – despite or even because of the current dramatic changes in pharmaceutical industry. Enjoy reading!

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Bert Klebl, (Dortmund)
Gerhard Müller, (Planegg)
Michael Hamacher, (Dortmund)

Part One
Hit Finding and Profiling for Protein Kinases: Assay Development and Screening, Libraries

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1
In Vitro Characterization of Small-Molecule Kinase Inhibitors

Doris Hafenbradl, Matthias Baumann, and Lars Neumann

1.1
Introduction

Typically the starting point for an early-stage drug discovery project is the identification of a small-molecule entity that, among other characteristics, has inhibitory activity against a given target kinase. Kinase inhibitors are usually identified in a high-throughput screening (HTS) campaign. The identified "hits" are selected on the basis of their inhibitory potential against the target kinase, the intellectual property situation around the small-molecule inhibitor class, the potential for further chemical optimization, and other criteria. Once the optimization process has started, several parameters have to be considered and continuously monitored. In most drug discovery programs, the optimization of the inhibitory activity of the small molecules against the target kinase represents the center of activities. While this parameter seems to be a straightforward and measurable parameter, there are a variety of possibilities of how an inhibitor might be binding to a protein kinase. Potentially, these different binding modes can cause modifications of the kinetic binding behavior of the compound. For the full assessment of an inhibitor, a detailed analysis of binding modes and kinetic consequences is required.

The optimization of a specific protein kinase inhibitor requires the constant assessment of a wide range of kinases to reduce the risk of possible side effects. It is therefore important to use comparable conditions in each protein kinase assay.

A successful drug candidate also requires a balanced physicochemical profile that determines the pharmacokinetic (PK) behavior of a small-molecule inhibitor in animals. In the past 10 years, a variety of *in vitro* assays have been developed and proven to be useful for the prediction of the PK parameters of an inhibitor.

Here, we describe in detail a selection of *in vitro* assays that are critical for the optimization process of small-molecule kinase inhibitors. For an appropriate start, a thorough optimization of the biochemical kinase assay is needed. In addition, one needs to consider the mode of inhibition and should be prepared for unexpected exceptions from the general rules. Besides the rationalization of the measurement of the biochemical activity and the selectivity that influence the pharmacodynamic behavior of a small-molecule inhibitor, we will give an in-depth overview of options

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for *in vitro* measurement of parameters that determine the pharmacokinetic behavior of small-molecule inhibitors.

1.2 Optimization of a Biochemical Kinase Assay

At the first glance, a biochemical kinase assay seems to be a very straightforward enterprise with only very few parameters that can be modified: the concentration of ATP, substrate, and protein kinase, the composition of the reaction buffer, and the reaction time. Nevertheless, a detailed optimization process is needed and several considerations have to be taken into account. In the following, we will give guidance for the evaluation of each step of the assay optimization and how this information is used to achieve the goal: a biochemical screening assay that yields reliable and reproducible information about the inhibitory activity of a small molecule as one of the most critical parameters throughout an entire drug discovery project. The optimization process for the AGC kinase Rock II is used as an example for describing in detail the considerations and evaluation of the results.

1.2.1 Step 1: Identification of a Substrate and Controlling of the Linearity between Signal and Kinase Concentration

Finding a substrate that is recognized and efficiently phosphorylated by the kinase of interest is the first essential step in developing a biochemical kinase assay. Equally important is the identification of the kinase concentration to start the assay optimization that guarantees a sufficiently high signal and at the same time good linearity between signal and kinase activity.

When the concentration of kinase is low, the concentration changes in ATP, ADP, and phosphorylated substrate are very small after a given reaction time. As a consequence, the associated assay signal is low and inaccurate (Figure 1.1, region of low assay signal). At moderate kinase concentrations, a sufficiently high assay signal can be detected and at the same time linearity between signal and kinase activity is observed. Thus, for example, a doubling of kinase activity is directly translated into the doubling of the assay signal (Figure 1.1, linear region). At high kinase concentrations, the bulk of ATP or substrate transforms into phosphorylated substrate after the given reaction time and the linearity between kinase activity and assay signal is lost (Figure 1.1, nonlinear region). At very high kinase concentration, all ATP or substrate is converted into ADP and phosphorylated substrate and an even higher kinase concentration cannot increase the signal further (Figure 1.1, insensitive region). Thus, as soon as the assay is depleted of either ATP or substrate, the assay is blind to changes in kinase activity. This situation is detrimental for two reasons. First, if the goal is to improve the assay conditions in order to increase the kinase activity, the assay cannot deliver an answer since changes in the kinase activity do not translate into a change in signal. A further increase in kinase activity cannot be

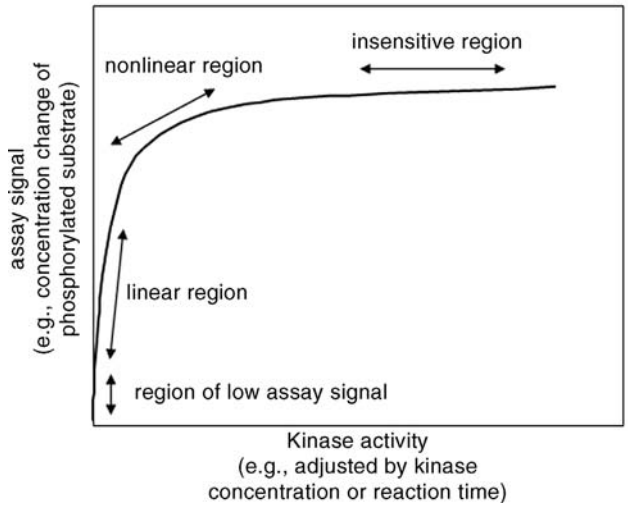

Figure 1.1 Assay signal is plotted against kinase activity. The assay signal can be derived from the concentration change of ATP, ADP, or phosphorylated substrate after the given reaction time. Kinase activity is adjustable, for example, by varying the kinase concentration or reaction time. The plot is separated in four distinct regions. (1) Region of low assay signal at very little kinase activity. In this region, the kinase activity is so low that only very little concentration changes in either educts or product have occurred. Usually, this region yields signals that are too weak to generate reliable data. (2) Linear region. At higher kinase activities, the concentration changes are larger and therefore the assay signals are generally strong enough to give robust data quality. This region is the optimal to perform kinase assays since a change in kinase activity is translated linearly in a signal change. (3) Nonlinear region. At even higher kinase activities, most of the ATP and/or substrate is transformed into ADP and phosphorylated substrate, respectively. In this region, high assay signals can be achieved, but kinase activity and signal do not depend linearly on each other anymore. (4) Insensitive region. If all ATP or substrate is consumed after the investigated reaction time, the maximal possible change of signal has been reached. Increased kinase activity cannot modulate the signal anymore because ATP and/or substrate has been completely consumed. Thus, neither an increase nor a decrease in kinase activity can be detected. In this region, the assay is insensitive to both an improvement of kinase activity (e.g., by optimizing the buffer components) and the inhibition of kinase activity (e.g., by the presence of a kinase inhibitor) and should therefore be avoided implicitly.

detected because even less kinase activity is sufficient to consume all ATP or substrate. Second, in the opposite scenario the question is whether or not a compound reduces the activity of a kinase. If the compound blocks 50% of the kinase activity, no change of the assay signal can be detected, as even 50% kinase activity is sufficient to consume all ATP or substrate within the given reaction time. Therefore, the activity of an inhibitor would be underestimated or the inhibition would not be detected at all.

In the first assay optimization step, both the substrate that yields in the highest kinase activity and the kinase concentration that combines sufficient assay signal and signal linearity are identified. Therefore, a series of potential substrates are tested in

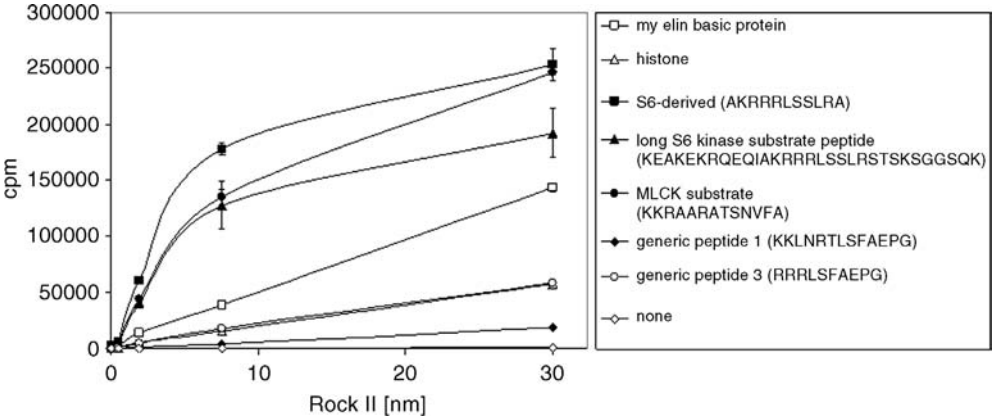

Figure 1.2 10 μM ATP and 12.5 μCi/ml ^{33}P-y-ATP are incubated with increasing concentrations of Rock II and 10 μM of various potential Rock II substrates in 40 μl 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT for 1 h at room temperature. After 1 h, the reaction was terminated by adding 10 μl 0.5 M EDTA. The reaction mixtures are transferred to phosphor cellulose filters and incubated with 60 μl 0.75% H_3PO_4 for 15 min. Remaining ^{33}P-y-ATP was removed from the filters by three washes with 200 μl 0.75% H_3PO_4 each. The filter-associated substrate-incorporated ^{33}P was quantified by scintillation counting and plotted against Rock II concentration. The error bars are given in standard deviations of duplicates.

the presence of increasing kinase concentrations. In Figure 1.2, step 1 of an assay optimization for the kinase Rock II is exemplified. Seven potential Rock II substrates were incubated with increasing concentrations of Rock II. In addition, Rock II was incubated in the absence of substrate. After 1 h, the reaction was terminated and the amount of phosphorylated substrate quantified. As shown in Figure 1.2, S6-derived peptide is phosphorylated most efficiently yielding the highest assay signal at low kinase concentrations. The generic peptide 3 was recognized with lowest efficiency. In the absence of substrate, consistently no assay signal was detected at all. In the presence of the S6-derived peptide, the linear assay region is found between 0.5 and 7 nM Rock II. Below 0.5 nM Rock II, only very small amounts of S6-derived peptide are phosphorylated and the assay signal is too small to be reliable. At Rock II concentration above 7 nM, the majority of the S6-derived peptide is phosphorylated and the assay reaches its nonlinear region. Thus, from step 1 the following information can be taken into account for the next optimization step: (1) S6-derived peptide is selected to be the substrate that is recognized most efficiently and (2) for the next optimization step, a Rock II concentration of 0.5 nM should be used to guarantee strict linearity between assay signal and kinase activity.

1.2.2 Step 2: Assay Wall and Optimization of the Reaction Buffer

In the second assay optimization step, a reaction buffer is identified that enables the kinase to work at its maximal capacity. In other words, the reaction buffer is