Handbook of Experimental Pharmacology

Volume 167

Inhibitors of Protein Kinases and Protein Phosphates

Contributors

D.R. Alexander, H.S. Andersen, N.R. Banner, R. Battistutta, A.M. Berghuis, S.M. Blake, D. Bossemeyer, C. Breitenlechner, D. L. Burk, A. Cheng, P. Cohen, S.W. Cowan-Jakob, B.J. Druker, R. Engh, D. Fabbro, G. Fendrich, D.H. Fong, P. Furet, M. Gaßel, J.D. Griffin, V. Guez, S. Herrero, H. Hidaka, R.E. Honkanen, L.F. Iversen, C.B. Jeppesen, S. Kumar, C. Kunick, C. Lampron, D.S. Lawrence, M. Leost, O. Lozach, H. Lyster, P.W. Manley, L. Meijer, J. Mestan, T. Meyer, N.P.H. Møller, S. Sarno, Y. Sasaki, S. Schmitt, M. Shibuya, Y. Suzuki, M.L. Tremblay, N. Uetani, A. Wakeling, M.H. Yacoub, G. Zanotti

Editors

Lorenzo A. Pinna and Patricia T.W. Cohen

Springer

Professor
Lorenzo A. Pinna
Università di Padova
Dipartimento di Chimica Biologica
Via Trieste 75
Padova 35121
Italy
e-mail: lorenzo.pinna@unipd.it

Patricia T. W. Cohen, Ph. D.
Professor of Molecular Biology,
MRC Protein Phosphorylation Unit,
School of Life Sciences
University of Dundee
MSI/WTB Complex, Dow St.
Dundee, DD1 5EH
Scotland, U. K.
e-mail: p.t.w.cohen@dundee.ac.uk

With 95 Figures and 17 Tables

Library of Congress Control Number: 2004103428

ISSN 0171-2004
ISBN 3-540-21242-6 Springer Berlin Heidelberg New York

Springer-Verlag is a part of Springer Science+Business Media
springeronline.com

Printed in Germany

Editor: Dr. R. Lange, Heidelberg, Germany
Desk Editor: S. Dathe, Heidelberg, Germany
Production Editor: H. Schwaninger, Heidelberg, Germany
Cover design: design & production GmbH, Heidelberg, Germany
Typesetting: Stürtz GmbH, Würzburg, Germany

Printed on acid-free paper 27/3150 hs – 5 4 3 2 1 0

Preface

Nearly all aspects of cell life (and death) are controlled by the phosphorylation of proteins, which is catalysed by protein kinases (PKs) and reversed by protein phosphatases (PPs). The role of PKs can be likened to that of interpreters, who translate stimuli and signals into biochemical events. For this reason, PKs and PPs are themselves interlinked and highly regulated, forming complex communicative networks. Not surprisingly, therefore, the deregulation of PKs results in cell malfunction, eventually resulting in neoplastic growth and other diseases. This makes PKs attractive targets for drugs not only to combat cancer, but also for other global diseases, notably diabetes, inflammatory and infectious diseases, stroke, hypertension and Alzheimer's. Actually about half of all proto-oncogenes so far identified encode PKs, and oncogenesis frequently results from the activation and/or overexpression of PKs. For example, overexpression of the epidermal growth factor receptor tyrosine kinase is the cause of many cancers of epithelial cell origin. In other instances, however, the link of PKs with neoplasia is not so straightforward, and depends on defective interactions with cellular partners of PKs, susceptibility to particular metabolic conditions, abnormal levels of other regulatory components or the combination of several of these factors.

The attractiveness of PKs as targets is enhanced by the fact that they are enzymes, which are targetable molecules par excellence. Thus their biological activity can be turned off very easily and precisely by drugs that block the catalytic site. Virtually all PKs belong to the largest single family of enzymes, numbering over 500 and accounting for almost 2% of the proteins encoded by the human genome. They share similar catalytic domains that catalyse the transfer of phosphate from ATP to serine, threonine or tyrosine residues in key regulatory proteins. Nevertheless, the structures of the catalytic domains of PKs are sufficiently distinctive that it is possible to develop compounds that are highly selective for a particular PK. Even the highly conserved binding site for the substrate ATP is surrounded by structural elements with variable features that can be exploited for the design of specific inhibitors, and most of the PK inhibitors currently undergoing human clinical trials are of this type. Two PK inhibitors are already in clinical use for the treatment of cancers (Gleevec and Iressa), while another is the immunosuppressant of choice to prevent tissue rejection after organ transplantation (rapamycin). At least 30 other PK inhibitors are undergoing human clinical trials to treat cancers and other diseases. These have the potential to provide a significant impact

on the management of epithelial cancers, such as breast and lung cancer. The approval of Gleevec for the treatment of a form of leukaemia by the FDA in May 2001 and more recently for the treatment of stomach cancers was a landmark because it is the first drug to be developed by targeting specific PKs. Moreover, its spectacular clinical effects, with minimal side effects, have had an enormous impact on the pharmaceutical and biotechnology industry. As a result, PKs have become the second most important family of drug targets, 20%–30% of all drug development programmes now being concentrated in this area. Although most PK inhibitors currently under investigation as potential drugs are ATP site-directed ligands, the field is still in its infancy, and there is tremendous potential to develop different types of drugs that target the binding sites for the protein substrates or which prevent the activation of PKs, since many of these enzymes are arranged in 'cascades' in which one PK activates or inhibits another one. Longer-term strategies would involve approaches based on gene therapy in which the mutant PK would be replaced by the wild-type enzyme.

PPs have received less attention to date as potential drug targets than PKs. The empirical discovery of an immunosuppressant drug that revolutionised organ transplantation (ciclosporin) and the subsequent recognition that it is a specific inhibitor of one PP indicates that PPs can be effective drug targets. An anticancer agent also discovered empirically (fostriecin) is now recognised to be a PP inhibitor. Other PPs, such as PTP1B, are currently under active investigations as drug targets for the treatment of diabetes and other diseases. As with PKs, known PP inhibitors at present target the active site but since many PPs are complexes with regulatory subunits, there is a potential for developing drugs that target the binding site of these regulatory subunits or their interaction with regulators. Thus the expansion of PPs as suitable drug targets may eventually follow that of PKs.

This volume of HEP highlights the tremendous pharmacological potential of PK and PP inhibitors, by providing a thorough overview of the most remarkable achievements in the field and illustrating how beneficial these studies can be for the advancement of both basic knowledge on biological regulation and deregulation and for the clinical treatment of a wide spectrum of diseases.

List of Contributors

(Addresses stated at the beginning of respective chapters)

List of Contents

HEP (2005) 167:1–7

Protein Kinase Inhibitors for the Treatment of Disease: The Promise and the Problems

P. Cohen

Medical Research Council Protein Phosphorylation Unit,
University of Dundee, MSI/WTB Complex, Dundee, Scotland, DD1 5EH, UK
p.cohen@dundee.ac.uk

1 The Promise

The reversible phosphorylation of proteins, catalysed by protein kinases and phosphatases, was first identified as a regulatory device in the 1950s, and it has been established for many years that this control mechanism regulates most aspects of cell life. However, it was only in the 1990s that interest in developing inhibitors of protein kinases and phosphatases started to enter centre stage (see Cohen 2002a,b for historical reviews). The first two drugs shown to target these classes of enzyme were cyclosporin, an inhibitor of protein phosphatase 2B (PP2B, also called calcineurin) (Liu et al. 1991) and rapamycin, an inhibitor of the protein kinase mTOR (mammalian target of rapamycin) (Heitman et al. 1991), which are the immunosuppressants that have permitted the widespread use of organ transplantation. However, these drugs were developed and approved for clinical use before their mechanism of action was identified. Fasudil, an isoquinoline sulphonamide that inhibits several protein kinases with relatively low potency, such as the Rho-dependent protein kinases (ROCK) (Davies et al. 2000), was developed by Hiroyoshi Hidaka in the 1980s and approved in Japan in 1995 for the treatment of cerebral vasospasm. ROCK can constrict blood vessels by inhibiting smooth muscle myosin phosphatase, but whether the clinical efficacy of fasudil results from its inhibition of ROCK, another protein kinase(s) or a completely different target, is unclear. Current information about this drug is discussed by Hidaka et al. (in Part 4).

Glivec (also called imatinib and STI-571), developed by Nick Lydon and his colleagues at Novartis, was the first drug to be developed by targeting a specific protein kinase and was approved for clinical use in the USA in 2001. It targets the protein tyrosine kinase c-Abl, which is mutated to the constitu-

tively active BCR-Abl fusion protein in nearly all cases of chronic myelogenous leukaemia (CML). The spectacular efficacy and minimal side effects of Glivec, first highlighted by Brian Druker, resulted in the most rapid approval of a drug in FDA history and was a landmark event in this area. The development of Glivec and its implications for the future of drug discovery in this area are discussed by Fabbro et al. (in Part 4). Interestingly, Abl is not the only protein tyrosine kinase targeted by Glivec. It also inhibits the c-Kit receptor tyrosine kinase and the platelet-derived growth factor (PDGF) receptor. The c-Kit receptor is mutated to an abnormally active form in many gastrointestinal stromal tumours (GISTs) and the efficacy of Glivec for the treatment GISTs is equally impressive, resulting in its approval for this therapeutic use in 2002. The potential of Glivec to treat several types of cancer is discussed by Druker (in Part 4).

Following on from the successful launch of Glivec, Iressa a potent inhibitor of the epidermal growth factor (EGF) receptor tyrosine kinase was approved in Japan in 2002 and in the USA in 2003 for the treatment of some types of lung cancer. Developed by AstraZeneca, this drug is discussed by Wakeling (in Part 4). Drugs that inhibit the vascular endothelial-growth factor (VEGF) or fibroblast growth factor (FGF) receptor tyrosine kinases are undergoing phase III clinical trials and may be among the next protein kinase inhibitors to be approved for clinical use. VEGF and FGF play key roles in angiogenesis, and inhibitors of their receptors destroy the tumour's vascular supply. For this reason these compounds may be useful for the treatment of several types of cancer.

Compounds that inhibit protein serine/threonine kinases are also undergoing human clinical trials in a number of therapeutic areas. For example, at least four companies have inhibitors of p38 mitogen-activated protein (MAP) kinase in the clinic. These compounds suppress the production of tumour necrosis factor (TNF) and some other proinflammatory cytokines and show efficacy for the treatment of rheumatoid arthritis and other chronic inflammatory diseases. These programmes are discussed by Kumar and Blake (in Part 2). In the same section, Meijer (in Part 2) discusses inhibitors of cyclin-dependent protein kinases (CDKs), which are undergoing clinical trials as anti-cancer agents, and inhibitors of GSK3 which, although at the preclinical stage, have shown potential for the treatment of several diseases including type II diabetes (Cline et al. 2002; Ring et al. 2003) and stroke (Cross et al. 2001). Inhibitors of MAP kinase kinase 1 (MKK1, also called MEK) and RAF (product of the proto-oncogene *Raf*) are undergoing clinical trials as anti-cancer agents, and inhibitors of mixed lineage kinase 3 (MLK3) to prevent neurodegeneration (reviewed in Cohen 2002b). However, this is only the 'tip of the iceberg'. Over the past few years protein kinases have become the second most studied group of drug targets after G protein-coupled receptors, accounting for a quarter or more of drug discovery programmes

worldwide. The number of protein kinase inhibitors undergoing human clinical trials at the present time almost certainly exceeds 100.

The discovery that PP2B, a serine/threonine-specific protein phosphatase, was inhibited specifically by cyclosporin highlighted the potential of protein phosphatases as drug targets, and programmes to develop specific inhibitors of several of these enzymes are underway. Protein tyrosine phosphatase IB (PTP1B) appears to be one of the enzymes that dephosphorylates and inactivates the insulin receptor, because mice that do not express it are hypersensitive to insulin and maintain normal blood glucose levels at half the normal circulating of insulin (Elchebly et al. 1999). In addition, these mice do not become obese when fed a high-fat, high-carbohydrate diet. For these reasons, PTPIB is potentially an attractive target for the development of a drug to treat diabetes and/or obesity, as discussed by Cheng et al. (in Part 3). However, although interesting compounds have been developed that are relatively specific inhibitors of PTP1B, as discussed by Møller (in Part 3), no inhibitors of this enzyme appear to have entered clinical trials. CD45 is another protein tyrosine phosphatase that is potentially an attractive drug target, because it is only expressed in cells of the immune system and is essential for T cell activation. Inhibitors of CD45 therefore have the potential to be effective immunosuppressants, but may lack the side effects associated with cyclosporin and rapamycin whose targets (PP2B and TOR) are expressed in nearly all cells and tissues. This topic is discussed by Alexander (in Part 3).

A number of toxins and tumour promoters are potent inhibitors of several members of one of the major classes of protein serine/threonine phosphatases, termed the PPP subfamily. They include the marine toxins responsible for diarrhetic seafood poisoning (okadaic acid and related compounds) and the algal toxins that are a threat to water supplies (microcystins) (reviewed in MacKintosh and MacKintosh 1994). Indeed, microcystins are the most potent liver carcinogens known to man. One might therefore predict that compounds which inhibit the catalytic subunits of these protein phosphatases would frequently be oncogenic and of little use as therapeutic agents. However, as discussed by Honkanen (Part 3), both fostriecin and cantharidin, which inhibit the same protein phosphatases, are cytotoxic for tumour cells and have been tested in phase I human clinical trials as anti-cancer agents. Not surprisingly, there are a number of side effects associated with the use of these compounds, and it seems more likely that drugs will eventually be developed that disrupt the functions of protein serine/threonine phosphatases in more subtle and specific ways. For example, the ability of the serine/threonine-specific protein phosphatase 1 (PP1) to dephosphorylate many proteins is controlled by its interaction with a great variety of 'targeting' subunits that direct it to specific subcellular locations and confer unique regulatory properties upon it. The form of PP1 associated with liver glycogen, which dephosphorylates and activates glycogen synthase, com-

prises the catalytic subunit of PP1 complexed to a glycogen-targeting subunit G_L. The ability of the PP1–G_L complex to dephosphorylate glycogen synthase is prevented when the active form of glycogen phosphorylase (termed phosphorylase a) binds to the extreme C-terminus of G_L, providing a mechanism for inhibiting glycogen synthesis when glycogenolysis is activated and vice versa (Armstrong et al. 1998). A drug that prevented the interaction of phosphorylase a with G_L would have the potential to lower the concentration of glucose in the blood by activating glycogen synthase and so stimulating the conversion of glucose into liver glycogen.

1.1 The Problems

There are over 500 protein kinases encoded by the human genome, most of which are members of the same superfamily. This has created a plethora of potential targets that can be studied in a unified way, but has highlighted the difficulty in developing compounds that are capable of inhibiting one of these enzymes specifically. The development of Glivec has shown that inhibition of more than one protein kinase can sometimes be beneficial, allowing the same drug to have more than one therapeutic use. However, more frequently one would expect such a lack of specificity to give rise to unwanted or unacceptable side effects. The recent availability of large panels of protein kinases (e.g. Davies et al. 2000; Bain et al. 2003) has been of considerable help in assessing the specificities of protein kinase inhibitors, and it is to be expected that such panels will continue to expand and eventually include the entire repertoire of protein kinases.

Lack of specificity may also mean that the therapeutic effect of a drug is actually mediated by inhibition of another protein kinase and not by inhibition of the kinase for which it was originally developed. For example, inhibitors of the cell cycle regulator CDK2 have been developed that suppress the proliferation of tumour cells, but these compounds may actually exert their therapeutic effects by inhibiting other protein kinases, such as CDK7 and/or CDK9, which are regulators of RNA polymerase II. It is therefore unclear whether the effects of these compounds are really mediated via CDK2. In order establish that the therapeutic effect of a drug is mediated by inhibition of a particular protein kinase one needs to show that the effects of the drug disappear in cells that express a drug-resistant mutant of the protein kinase (Eyers et al. 1999). It is possible to convert protein kinases to drug-resistant forms by single amino acid replacements (Brown et al. 1995; Eyers et al. 1998) so that, as for other types of drug, the development of drug resistance is a potential hazard. Mutations in Abl that make it resistant to Glivec are the cause of relapse in patients with chronic myelogenous leukaemia (Gorre et al. 2001). However, resistance to Glivec is mainly seen in patients

with the most advanced stage of this disease, where extensive genomic instability has already taken place.

Most of the protein kinase inhibitors developed thus far target the ATP-binding site and must therefore be of sufficient potency to compete with the millimolar concentrations of ATP that are present in the intracellular milieu. Clearly, it is possible to develop compounds with the requisite in vivo potency, as shown by the number of compounds undergoing human clinical trials. However, this remains a challenging problem, especially for protein kinases that bind ATP particularly tightly. Some of the most interesting protein kinase inhibitors developed thus far, including Glivec (Schindler et al. 2000) and the p38 MAP kinase inhibitor BIRB 796 (Pargellis et al. 2002), not only target the ATP-binding site, but also trigger structural changes that induce the inactive conformations of these protein kinases. Two other compounds, PD 98059 and U0126, do not target the ATP-binding site at all, but bind to the inactive conformation of MKK1, preventing it from being activated by the protein kinase Raf (Alessi et al. 1995; Davies et al. 2000). The development of more compounds that prevent one protein kinase from activating another may be a promising strategy for novel drug development in this area, since many of these enzymes are components of protein kinase 'cascades'. Another way of generating compounds that are not ATP-competitive would be to target the binding sites for protein substrates, a topic discussed by Lawrence (in Part 1).

There are about 150 protein phosphatase catalytic subunits encoded by the human genome, and they fall into three main superfamilies. The generation of compounds that discriminate between different protein phosphatases is therefore also a challenging one. However, in contrast to protein kinases, the option of targeting an ATP binding pocket does not exist. Moreover, the protein substrate-binding cleft can be very polar, as in the case of PTP1B (Kellie 2003). This has made it difficult to develop compounds that combine high potency with cell permeability. The only protein phosphatase inhibitor that has advanced to human clinical trials, cyclosporin, inhibits PP2B in an unusual way; it binds to the protein cyclophilin, and the cyclosporin–cyclophilin complex then inhibits the protein phosphatase (Liu et al. 1991). As discussed earlier, it seems more likely that the future of drug discovery in this area may lie in targeting the regulatory subunits of serine/threonine-specific protein phosphatases.

Finally, it is important to mention that inhibitors of protein kinases are not only becoming important for the treatment of disease, but also as reagents for the study of cell signalling. The huge number of citations garnered by the publications that have introduced these compounds to the scientific community are a reflection of the widespread need for these compounds by the scientific community. For example, I was surprised to learn from the Institute for Scientific Information that the paper we published in 1995 with David Dudley and Alan Saltiel at Parke Davis on the mechanism

of action of PD 98059 (Alessi et al. 1995) was the UK's most frequently cited original research paper over the past 10 years in the fields of biology and biochemistry, while our publication with Peter Young and John Lee at SmithKline Beecham on the specificity of SB 203580 (Cuenda et al. 1995), a prototypic p38 MAP kinase inhibitor, was the UK's sixth most cited original research paper over this period. Although many compounds are advertised for sale as 'specific protein kinase inhibitors', in practice many have turned out to inhibit so many protein kinases that conclusions drawn from their use are likely to be erroneous (Davies et al. 2000; Bain et al. 2003). The number of really useful protein kinase inhibitors that are available commercially is still rather limited, but the number will increase considerably over the next few years. I believe that pharmaceutical companies have much to gain from the discoveries that will be made by exploiting these compounds, and it is to be hoped that many more will be released for general use in the future.

References

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR (1995) PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270:27489–27494

Armstrong CG, Doherty MJ, Cohen PTW (1998) Identification of the separate domains in the hepatic glycogen-targeting subunit of protein phosphatase 1 that interact with phosphorylase a, glycogen and protein phosphatase 1. Biochem J 336:699–704

Bain J, McLauchlan H, Elliott M, Cohen P (2003) The specificities of protein kinase inhibitors: an update. Biochem J 371:199–204

Brown EJ, Beal PA, Keith CT, Chen J, Shin TB, Schreiber SL (1995) Control of p70S6 kinase by kinase activity of FRAP in vivo. Nature 377:441–446

Cline GW, Johnson K, Regittnig W, Perret P, Tozzo E, Xiao L, Damico C, Schulman GI (2002) Effects of a novel glycogen synthase kinase-3 inhibitor on insulin-stimulated glucose metabolism in Zucker diabetic fatty (fa/fa) rats. Diabetes 51:2903–2910

Cohen P (2002a) The origins of protein phosphorylation. Nat Cell Biol 4:E127–E130

Cohen P (2002b) Protein kinases—the major drug targets of the twenty-first century? Nat Rev Drug Discov 1:309–315

Cross DA, Culbert AA, Chalmers KA, Facci L, Skaper SD, Reith AD (2001) Selective small molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurons from death. J Neurochem 77:94–102

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stress and interleukin-1. FEBS Lett 364:229–233

Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105

Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP (1999) Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase 1B gene. Science 282:1544–1548

Eyers PA, Craxton M, Morrice N, Cohen P, Goedert M (1998) Conversion of SB-203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino acid substitution. Chem Biol 5:321–328

Eyers PA, Van den Ijssel P, Quinlan R, Goedert M, Cohen P (1999) Use of a drug resistant mutant of stress activated protein kinase 2a/p38 to validate the in vivo specificity of SB203580. FEBS Lett 451: 191–196

Gorre ME, Mohammed M, Ellwood K, Hsu N, Pacquette R, Rao PN, Sawyers CL (2001) Clinical resistance of STI 571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 293:876–880

Heitman J, Mowa NR, Hall MN (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science 253:905–909

Kellie S (2003) Protein tyrosine phosphatases: potential roles in disease. In: Watling KJ (ed) Celltransmissions. Sigma-Aldrich, St Louis, pp 3–8

Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66:807–815

MacKintosh C, MacKintosh RW (1994) Inhibitors of protein kinases and phosphatases. Trends Biochem Sci 19:444–447

Pargellis C, Tong L, Churchill L, Cirillo PF, Gilmore T, Graham AG, Grob PM, Hickey ER, Moss N, Pav S, Regan J (2002) Inhibition of p38 MAP kinase by utilising a novel allosteric binding site. Nat Struct Biol 9:268–272

Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, Ma ST, Reeder JW, Samuels I, Slabiak T, Wagman AS, Hammond M-EW, Harrison SD (2003) Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilisation in vitro and in vivo. Diabetes 52:588–595

Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J (2000) Structural mechanism for STI 571 inhibition of Abelson tyrosine kinase. Science 289:1938–1942

Part I
General Aspects of PKs Inhibition

HEP (2005) 167:11–44

New Design Strategies for Ligands That Target Protein Kinase-Mediated Protein–Protein Interactions

D. S. Lawrence

Department of Biochemistry, The Albert Einstein College of Medicine,
1300 Morris Park Ave, Bronx, NY 10461, USA
lawrence@medusa.bioc.aecom.yu.edu

Abstract Protein–protein interactions serve as the molecular engine that drives the formation and disassembly of intracellular signaling pathways. Antagonists of these interactions could play key roles as both biological reagents and therapeutic compounds. However, much of the early work in this area with peptides revealed that these species, in general, bind with modest affinity to their protein targets. In addition, when these studies first commenced nearly 20 years ago, the technology for the intracellular delivery of peptides and modified analogs thereof was rudimentary. In the intervening years, not only has this technology dramatically improved, but the global role that protein–protein interactions play in transducing intracellular signals has become simply too obvious to ignore. With the introduction of combinatorial library methods, it is now a simple matter to identify consensus sequences recognized by protein interaction domains. An array of strategies has now been developed to transform these otherwise modest binding consensus sequences into high-affinity ligands. These strategies include the design of high-affinity replacements for key amino acid residues in consensus peptides, the construction of

multidomain-binding peptides, and the structural modification of consensus sequence peptides. In several of these instances, unprecedented affinity (<nM) and selectivity (>1,000-fold versus closely related protein targets) have been achieved.

Keywords Signal transduction · Antagonists of protein–protein interactions · Peptide-based inhibitors · Protein kinases and phosphatases · Combinatorial libraries · Amino acid analogs · Bivalent inhibitors · Structurally modified peptides

1 Introduction

Protein–protein interactions serve as the adhesive that drives the assembly of signaling pathways. However, this adhesive is transient in nature. Once the cell has acknowledged the environmental stimulus, signaling pathways must rapidly disassemble to restore the cell to its resting state. At first glance, agents that selectively target key protein–protein interactions would appear to serve as ideal inhibitors of cell signaling as well as potential therapeutics. First, protein–protein interactions are typically exemplified by well-defined consensus sequences, which can often be reasonably selective for a given protein–protein pair. Consequently, the preparation of inhibitors of protein–protein interactions appears, at least on paper, to be reasonably straightforward since, the acquisition of preferred consensus sequences employs simple and well-defined methods. Second, the intracellular levels of protein–protein-binding partners rarely surpass low micromolar amounts, thereby rendering competition with endogenous substrates relatively unimportant. In spite of these apparent advantages, the overwhelming majority of reported protein kinase inhibitors target the ATP-binding site, a region common to all protein kinases, non-protein kinases, and many other ATP-binding proteins. Furthermore, the intracellular concentration of ATP (~1–10 mM) is much larger than its K_m (serine/threonine kinases ~1–10 μM; tyrosine kinases ~20–50 μM), which all but assures that the ATP-binding site will be saturated with ATP. The consequence of the latter is that inhibitors that target the ATP-binding site must be present at intracellular concentrations that significantly exceed their in vitro-determined K_i values. Finally, the acquisition of ATP analogs that specifically target individual protein kinases requires the initial screening of a large starting library of potential inhibitor candidates. This is then followed by a substantial synthetic effort that involves the preparation of secondary and tertiary libraries based on initially identified leads. The notion of disrupting signaling pathways via antagonists of protein–protein interactions has been unpopular for a number of reasons, including issues related to potency, intracellular stability and uptake, and general bioavailability (i.e., with respect to therapeutics). However, recent advances in various delivery technologies coupled with our in-

creasing understanding of the widespread participation of protein-binding domains in signaling, has led to a renewed interest in the development of anti-signaling agents that disrupt intracellular protein–protein interactions.

Given the long-dormant state of this field, which is characterized by a recent reawakening, a broad overview of the general area of protein kinase-mediated protein–protein interactions and their corresponding antagonists is provided. This includes a summary of the methods employed to obtain consensus sequence information, a general synopsis of protein-binding domains, and finally a description of antagonists of protein–protein interactions as well as emerging strategies to acquire ever more potent and selective inhibitory agents.

2 Identification of Consensus Sequences

2.1 Degradation of Protein Ligands

Amino acid recognition sequences that drive protein–protein interactions were initially identified via partial digestion of one of the protein-binding partners. Fragments that were determined to retain binding potency were then sequenced. Further refinement of the amino acid recognition sequence could then be explored via the preparation of synthetic peptides. This strategy is best exemplified by the work described in the 1980s on the potent "heat-stable" inhibitor of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) known as PKI (protein kinase inhibitor). Krebs, Walsh, and their colleagues (Scott et al. 1985a,b; Cheng et al. 1986; Scott et al. 1986; Van Patten et al. 1986; Glass et al. 1989) identified a series of peptides that serve as extraordinarily potent inhibitors (K_i<50 nM) of PKA. Protease digestion of the isolated protein furnished a 20-mer peptide that acts as a competitive inhibitor versus peptide substrate with a K_i in the subnanomolar range. These investigators demonstrated that the sequence Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile is the active site-directed component of PKI, where the Ala residue is positioned at the site normally reserved for the phosphorylatable serine. Indeed, subsequent studies demonstrated that insertion of serine in active site-directed sequences derived from PKI generates powerful peptide substrates (Mitchell et al. 1995). However, the new library-based methods introduced in the 1990s have largely supplanted the biochemical approaches for identifying amino acid sequences recognized by protein interaction domains. The new methodologies are not only significantly less labor intensive than their classical counterparts, but are also able to bypass the need for large quantities of both binding partners (for digestion and sequencing purposes).

2.2 Synthetic Peptide Libraries

A large number of different library strategies using synthetic peptides have been described. These approaches include one-bead/one-peptide libraries (Wu et al. 1994), solution mixtures of peptides (Songyang et al. 1994), one-well/one-peptide strategies (Lee and Lawrence 1999), peptides on chips (Houseman et al. 2002), and even proteins on chips (Zhu et al. 2000). A detailed description of the vast array of peptide library strategies now available is well beyond the scope of this review. However, all of these methods offer a rapid means to quickly identify preferred amino acid sequences in what is typically a single experiment. Peptide-based libraries also permit the use of amino acid derivatives beyond the standard genetically encoded residues (e.g., post-translationally modified residues such as phosphoTyr, hydroxyPro, etc.). In addition, many of the methods not only identify a preferred consensus sequence, but also often furnish an assessment of the range of residues permitted at a given position on the peptide ligand.

Each of the peptide library strategies enjoys certain advantages while enduring specific disadvantages:

1. One-bead/one-peptide libraries are extremely easy to prepare via split-and-pool synthesis (Lam et al. 2003). However, these libraries are commonly composed of a mixture of millions of beads, with each bead possessing a unique peptide sequence. Consequently, a screening method must be devised so that the bead containing the tightest binding ligand can be readily identified. Possibilities include the use of a target protein that contains an appended fluorophore or is conjugated to an enzymatic reporter. Beads can also be identified via the introduction of radioactivity (i.e., the use of [γ-^{32}P]ATP). Once leads have been identified, the beads are isolated and the bound peptides identified by microsequencing. Given the heavy reliance upon the latter, the use of uncommon hypermodified residues is severely restricted.
2. Soluble peptide library mixtures have also been utilized to identify consensus sequences (Songyang and Cantley 1998). These libraries are prepared by treating the growing peptide chain with a mixture of the standard amino acid derivatives. The actual ratio of the amino acids introduced during the coupling reaction is based upon the relative coupling efficiencies of the individual residues. Consequently, a particular residue that couples sluggishly (e.g., Arg) is present at a greater relative ratio than one that couples readily (e.g., Gly). Following completion of the synthesis, the peptide mixture is cleaved from the resin and subsequently employed for consensus sequence identification. The latter is achieved by selective enrichment of the binding sequence, often using an affinity column. For example, protein kinase-catalyzed phosphorylation of the mixture is allowed to proceed until a small fraction (<1%) of the total peptide is phosphorylated. The phosphopeptide

mixture is subsequently isolated and sequenced as a mixture. Each position on the peptide is not identified as a single residue, but rather as the relative abundance of all the amino acid residues at a particular site. The residue present in the largest amount at a given position is taken as the one most favored at that site. However, since a peptide mixture, as opposed to a single peptide, is sequenced, this strategy does not yield sequences of unique peptides but merely determines the preferences for particular residues at specific positions. An inherent assumption of this method is that selection at each position is independent of the adjacent amino acids. Consequently, this technique ignores the possibility that two or more residues can act in a synergistic fashion to promote target protein affinity.

3. The one-well/one-peptide approach ("parallel synthesis") (Granier 2002) employs pure peptides that are spatially segregated from one another (Lee and Lawrence 1999). This technique has the advantage that the sequence of each peptide in each well is verified in advance. Furthermore, a wide assortment of hypermodified amino acid residues can be employed, since the synthesis history of each peptide in each well in known. An obvious disadvantage is that the size of these libraries, by necessity, is much smaller than those described in points *1* and *2* above. Variations that employ spatially segregated mixtures ("positional scanning") have been reported that address this concern (Houghten et al. 1996).
4. Peptide chips represent the solid phase version of the method described in *3* (Houseman et al. 2002). The added advantage of this system is the higher spatial density, and therefore smaller chip size [membranes have been employed in this technique as well (Frank 2002)]. However, the increased spatial density of the individual peptide "colonies" can come at a cost. Although methods that employ fluorescence detection of target protein binding will work well in this system, other common methods, such as those that utilize radioactivity, cannot be applied to ultra high-density chips.

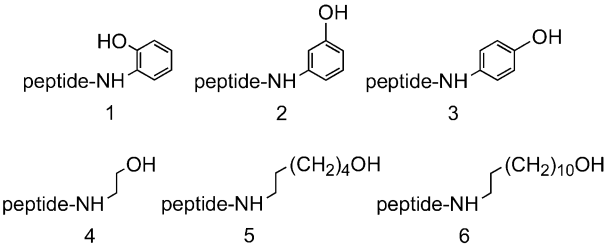
2.3 Phage Display

Phage display is a genetically encoded peptide library strategy (Scott and Smith 1990; Smith and Scott 1993). In brief, peptides are displayed on the capsid protein of filamentous phage. Each virion particle displays a unique peptide sequence on its surface. Millions of phage clones are exposed to the protein target of interest and the affinity purified particles then amplified in *Escherichia coli*. Subsequent rounds of selection furnish a few "lead" clones, from which the displayed sequences can be determined via sequencing of the viral DNA coding region. Phage display has been used to acquire peptide ligand sequences for a wide variety of protein interaction domains (Smothers et al. 2002). The obvious limitation here is that the genetic basis for this method restricts the range of amino acids to the 20 standard residues.

3
The Protein-Binding Domains of Protein Kinases

Protein kinases are, first and foremost, catalysts that promote the transfer of a phosphoryl group from ATP to the acceptor hydroxyl moiety of serine, threonine, and/or tyrosine. The serine, threonine, and tyrosine residues must be embedded within the proper amino acid sequence in order to be recognized by a given protein kinase, a fact exemplified by the large number of synthetic peptide-based substrates that have been devised for scores of protein kinases. Of all the protein-binding domains contained within protein kinases, the active site region displays the greatest diversity in terms of sequence recognition. However, since all members of the protein kinase family utilize the same phosphoryl donor (ATP) and acceptors (serine, threonine, tyrosine), it is perhaps not too surprising that the conformation of the active site region is remarkably well-conserved (Johnson et al. 1998; Huse and Kuriyan 2002). The "protein kinase fold" is composed of two separate lobes, commonly designated as the N- and C-terminal lobes. The former is the smaller of the two and is composed of five antiparallel β-strands and a single α-helix. The larger C-terminal lobe is primarily α-helical in structure. ATP resides in a cleft that lies at the interface between the N- and C-terminal lobes. By contrast, the peptide/protein phosphoryl acceptor is primarily associated with the C-terminal lobe. The catalytic domain of protein kinases can assume active and inactive conformational states. The lobes in the former migrate toward one another, thereby closing the active site and promoting catalysis.

Protein kinases are commonly differentiated on the basis of their preferred phosphoryl acceptor group on the protein substrate: either the aliphatic hydroxyl moieties of serine and threonine ("serine/threonine protein kinases") or the aromatic phenol of the tyrosine residue ("tyrosine protein kinases"). A few protein kinases display the property of "dual specificity" in terms of their ability to recognize and phosphorylate both aliphatic and aromatic alcohols on peptides or proteins in vitro (fewer still display this property in living cells) (Dhanasekaran and Premkumar Reddy 1998; Marin et al. 1999). However, in a very strict sense, the segregation of protein kinases into these separate camps most likely has less to do with the protein kinases themselves and more to do with the fact that the genetic code is limited to only 20 different amino acids. For example, PKA, a well-known serine/threonine-specific protein kinase, phosphorylates appropriately designed aromatic alcohols (e.g., **1**–**3**) (Lee et al. 1994).



Furthermore, Src, an equally well-established tyrosine-specific protein kinase, phosphorylates aliphatic alcohols (e.g., **4–6**) (Lee et al. 1995a,b). Obviously, from the biological point of view, compounds **1–6** are mere curiosities. However, in terms of designing sensors, substrates, and antagonists of protein kinase-mediated protein–protein interactions, derivatives **1–6** are an important reminder that, unlike cells, chemists are not limited to the standard 20 amino acids fixed by the genetic code. Indeed, early work with consensus sequence peptides *containing conventional amino acids* is, in large part, responsible for the prevailing notion that inhibiting signaling pathways via disruption of protein–protein interactions is a strategy doomed to failure.

In addition to the "active site specificity" (i.e., serine/threonine versus tyrosine) of protein kinases, these enzymes display a preference for the amino acid sequence that encompasses the phosphorylatable residue (the "sequence specificity"). Compilations of sequences phosphorylated by protein kinases are available and these will not be recapitulated here (Pinna and Ruzzene 1996). However, certain trends are apparent:

1. The overwhelming majority of protein kinases will also phosphorylate simple peptides, thereby rendering the in vitro assay of these enzymes fairly straightforward. In addition, this demonstrates that the protein-binding region of these enzymes is sufficiently structurally well established to recognize substrates on its own (i.e., large intact protein substrates are not required for the protein kinase to assume an active state). However, the notion that the protein-binding region is the only site on the protein kinase that is responsible for substrate specificity is decidedly untrue.
2. Absolute protein kinase specificity is not encoded within the substrate-binding site. In other words, the consensus sequence surrounding the phosphorylatable residue, although an important parameter of protein kinase recognition, is not the sole determinant of specificity. For example, the cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and PKA, members of the same protein kinase subfamily, display largely overlapping specificities with respect to simple peptide substrates (Mitchell et al. 1995; Wood et al. 1996). Certain protein kinase C (PKC) isoforms likewise phosphorylate the same peptides as PKA and PKG; however, the sequence preference of PKC is broad enough that PKC peptide substrates have

been devised that are recognized by neither PKA nor PKG (Yan et al. 2000). In short, it is unlikely that a peptide composed of only conventional amino acids will serve as an absolutely specific substrate for any given protein kinase. Indeed, Cohen and his colleagues have used this notion to generate a small set of peptides that serve as general substrates for more than three dozen different protein kinases (Ross et al. 2002).

3. Consensus sequence-containing active site-directed peptides are generally poor inhibitors. This fact is responsible for much of the common belief that targeting the substrate recognition site in particular, and protein–protein interactions in general, is an untenable strategy. Perhaps the best-known example is kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly, which serves as an excellent substrate for PKA. The K_m for this peptide is less than 20 μM, a value that was (incorrectly) taken as a reflection of the binding constant of the peptide. However, the corresponding nonphosphorylatable peptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly, is an exceedingly poor PKA inhibitor (K_i=320 μM) (Whitehouse et al. 1983). Much of the early discussion concerning the ineffectiveness of the Ala-substituted peptide centered on the possibility that the serine hydroxyl group (missing in the inhibitor) promotes binding affinity by two orders of magnitude. However, subsequent detailed enzymological studies revealed that the K_m value is a complex parameter that is dependent upon more than just the microscopic rate constants that control the active site association and dissociation of peptide substrate (Adams and Taylor 1992). In an analogous vein, poor inhibitors of tyrosine kinases (in which the phosphorylatable tyrosine residue was replaced with a phenylalanine) have been noted. Nevertheless, a few exceptions to the "rule" that active site-directed peptides serve as poor inhibitors are known. A naturally occurring "heat stable" protein-based inhibitor (PKI) of PKA is a powerful inhibitor (K_i<1 nM) (Whitehouse and Walsh 1983). A peptide fragment of PKI, most notably Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Leu-Gly-amide (where the Ala represents the site that would be reserved for serine) was reported to have a K_i of 36 nM (Glass et al. 1989). As an aside, the K_i for this peptide was originally determined under conditions of low salt; when the inhibitory potency of this peptide was subsequently reexamined several years later under more physiologically conditions, the K_i was found to be 500 nM (Wood et al. 1998). Nevertheless, the latter value does suggest that it is feasible to devise reasonably potent inhibitors based on standard amino acid residues alone. Indeed, perhaps the most outstanding example of this is the extraordinarily potent 24 amino acid-containing PKG selective inhibitor Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Typ-Lys-Lys-Leu-Arg-Lys-Lys-Lys-Lys-Lys-His (Dostmann et al. 2000). This peptide is likely engaged in interactions beyond the immediate vicinity of the active site. Indeed, there appears to be a general consensus of opinion that, unlike targeting the ATP-binding site, effective inhibitors of protein–protein interactions must coordinate to a relatively large surface area.

In addition to the active site, there are several other protein interaction domains that are commonly affixed to protein kinases. These include the SH2 and SH3 domains, which are prevalent among the tyrosine protein kinases. PDZ, LIM, WW, PTB, and others are found in many protein kinases and/or in the adaptor proteins that help to transduce the activity of these enzymes. In addition, protein kinases themselves serve as ligands for protein interaction domains present on anchoring proteins. Indeed, peptide-derived inhibitors that bind to these anchoring proteins and thereby block protein kinase docking, have been described (Csukai and Mochly-Rosen 1999). The primary focus of this chapter is on the acquisition of active site-directed peptide-based inhibitors and the emerging strategies to acquire ever more potent and selective agents.

4
Strategies for the Acquisition of Potent and Selective Peptide-Based Inhibitors of Protein Kinases

Although a few exceptions are known, in general, conventional peptides display modest affinities and poor selectivities for the protein interaction domains contained within protein kinases. Biological systems appear to have little need for high-affinity ligands for active sites, SH2, SH3, LIM, PDZ, and other protein-interaction domains due to the transient nature of signaling pathways. However, it is abundantly clear that biological systems have mastered the issue of selectivity. Selective expression of only certain protein kinases in specific cell types, or at precise intervals during the lifetime of the cell, offers one means to navigate the tricky waters of intracellular selectivity. Spatial segregation of protein kinases to specific intracellular sites represents another means by which selectivity can be achieved. Finally, given the comparatively large size of these proteins, and their correspondingly well-defined structures, selectivity may simply be attained via a highly precise three-dimensional choreography of interactions between binding partners. Consequently, the design of potent and selective artificial antagonists of protein–protein interactions represents a significant challenge, albeit an exciting one. The primary advantage enjoyed by the chemist is that he or she is not restricted to the 20 standard amino acids designated by the genetic code.

The acquisition of agents that target protein–protein interaction sites has the potential to be relatively straightforward. Consensus sequences are easy to identify. However, it is necessary to develop the tools and/or strategies that can convert peptides containing these sequences into agents that recapitulate the high selectivities observed in biochemical pathways while significantly surpassing the affinities that intracellular binding partners display for one another. The tools and strategies to achieve the twin goals

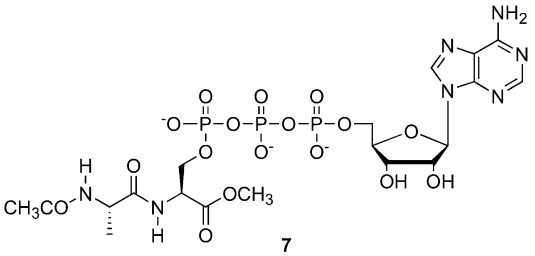
of potency and selectivity, within the framework of relatively small ligands (cf., proteins), are outlined below in three separate, but interrelated sections.

4.1 Mimetics of Key Residues in Consensus Sequence Peptides

4.1.1 Serine Analogs

As noted above, one of the first indications that the acquisition of effective peptide-based inhibitors for protein kinases might be problematic was the replacement of the phosphorylatable serine residue in the PKA substrate kemptide with an alanine to create a dead-end inhibitor. The latter proved to be an unexpectedly weak inhibitory agent (K_i>300 μM versus the K_m for kemptide <20 μM). One of the explanations offered for the low affinity, namely loss of the hydroxyl serine side chain as a potential hydrogen bond donor, was subsequently shown to be incorrect. However, this notion does suggest that there may be ways to improve upon the use of alanine as a non-phosphorylatable replacement for serine.

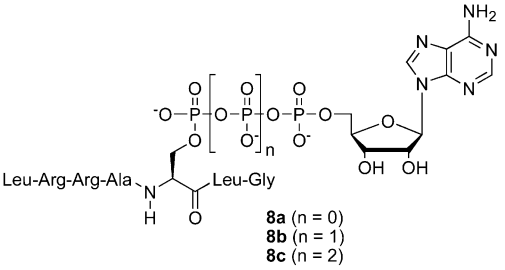
Coward and his colleagues were the first to suggest that the phosphorylatable residue in an active site-directed peptide could be substituted with an analog that is able to also engage the ATP-binding site (i.e., a bisubstrate inhibitor) (Lashmet et al. 1983).

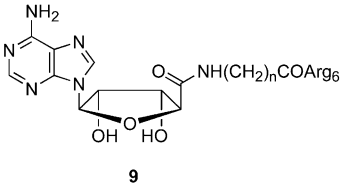


7

Although the ATP-γ-Ala-Ser ester **7** does not possess the requisite peptide framework for it to serve as a protein kinase inhibitor, it is a model of the type of compounds that were eventually prepared more than a decade later.

Gibson and his colleagues were the first to report the synthesis of adenosine phosphopeptides in a solid phase format (Medzihradszky et al. 1994).

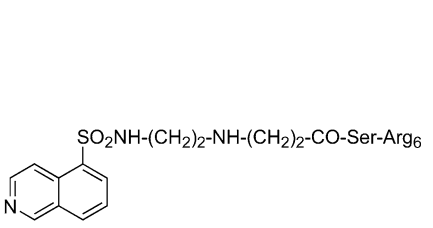




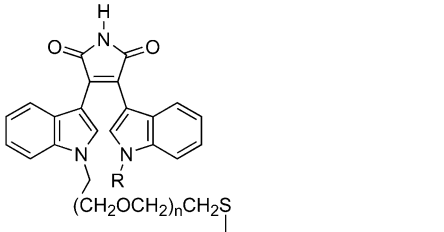
These investigators described the preparation and characterization of several analogs (**8**) of kemptide. Although the IC_{50} values of **8a**, **8b**, and **8c** (935 μM, 226 μM, and 68 μM, respectively) are modest, these inhibitors are significantly more effective than the simple Ala-containing analog Leu-Arg-Arg-Ala-Ala-Leu-Glu. The authors found that **8** displays a competitive inhibition pattern versus variable ATP, but such a pattern was not observed with respect to variable peptide substrate. One might expect that a bisubstrate analog would exhibit competitive patterns versus both ATP and phosphorylatable peptide. However, the absence of double competitive behavior does not necessarily rule out the two-site binding model. Strictly speaking, competitive behavior is observed for an inhibitor only if that inhibitor and the corresponding substrate bind in a mutually exclusive fashion to the *same enzyme form*. PKA is known to exhibit a primarily ordered mechanism with ATP binding first (Whitehouse et al. 1983). Consequently, one would expect compound **8** and ATP to associate with the same enzyme form, namely the free enzyme, and thereby exhibit competitive behavior. By contrast, given the nature of the ordered mechanism, peptide substrate preferentially coordinates to the enzyme–ATP complex, which would thereby rule out a competitive pattern with **8**.

Recently Uri and his colleagues have described a series of bisubstrate analogs that dispenses with the phosphoric anhydride portion of the ATP moiety (Loog et al. 1999). These investigators employed an adenosine-5′-carboxylic acid derivative as the ATP mimic which, using a variety of linkers, was appended to the N-terminus of an arginine rich peptide (**9**). The most effective analogs displayed IC_{50} values of between 100 and 300 nM for PKA and PKC. These bisubstrate analogs have been used to affinity-purify protein kinases (Loog et al. 2000). In addition, membrane-permeable fluorophore-labeled bisubstrate derivatives have been prepared (Uri et al. 2002; Viht et al. 2003).

In a departure from the ATP-based bisubstrate strategy, Sergheraert and colleagues designed (ATP mimics)-linker-substrate analogs (Ricouart et al. 1991). Isoquinoline and naphthalene sulfonic acid derivatives served as ATP replacements. The most potent of the several derivatives prepared was compound **10**.



10

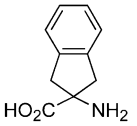


11

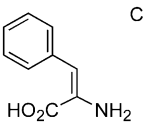
The latter exhibits a 25-fold selectivity in favor of PKA (K_i=4 nM) versus PKC (K_i=100 nM). The inhibitory potency of this derivative is impressive when one considers the fact that the ATP mimic alone is a nearly three orders of magnitude poorer inhibitor than **10**. However, since the ATP analog is appended off the N-terminus of the peptide, an unanswered question is the nature of the requisite structural requirements to replace a serine moiety that is contained within the interior of a consensus sequence. Finally, **10** acts as a competitive inhibitor versus variable ATP, but is not competitive with respect to variable peptide substrate. Sasaki, Maeda, and their coworkers likewise utilized an ATP analog (a bisindolylmaleimide) to prepare a series of bisubstrate inhibitors **11** that are designed to target the cyclin-dependent protein kinase, cdc2 (Sasaki et al. 1998). The best inhibitors display IC_{50} values in the low micromolar range (where X=no amino acid). However, when X=Ser, the inhibitory potency is reduced by two orders of magnitude.

4.1.2 Tyrosine Analogs

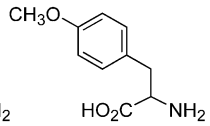
In an analogous vein to serine/threonine protein kinases, peptide-based inhibitors of tyrosine kinases were initially prepared by substituting the phosphorylatable tyrosine with the nonphosphorylatable phenylalanine. In general, the phenylalanine-for-tyrosine replacement generates exceedingly poor inhibitory agents (K_i>1 mM).



12



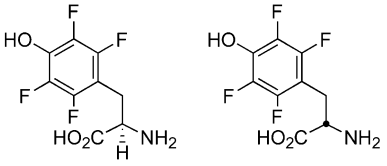
13



14

Several peptides containing tyrosine analogs (**12–14**) were reported in the 1980s, but these derivatives proved to be ineffective as inhibitors (Wong and Goldberg 1984; Shoelson et al. 1989).

Graves and his colleagues described the first example of an effective peptide-based tyrosine kinase inhibitor (Yuan et al. 1990).



15 **16**

The inhibitory agent, which contains the tetrafluorotyrosine moiety **15**, targets the insulin receptor with a K_i of 4 μM. The rationale for the use of the fluorinated tyrosine analog was based on the presumed mechanism of catalysis. These investigators reasoned that an active site base partially removes the aromatic hydroxyl proton during the transition state of the enzyme-catalyzed phosphoryl transfer reaction from ATP to the acceptor phenol. Presumably, the enzyme stabilizes this partial-negative charge on the phenol/phenoxide during the transition state, which suggests that a tyrosine analog that is negatively charged might be well accommodated within the active site. The four fluorine substituents not only lower the pK_a of the phenol, thereby promoting ionization to the phenoxide at physiological pH, but in addition they render the phenoxide less nucleophilic than its natural counterpart. These investigators also prepared the corresponding d-analog **16**, which also displays promising inhibitory activity (K_i=20 μM). Interestingly, although both **15** and **16** serve as competitive inhibitors versus variable peptide substrate, the l-analog directly competes with ATP as well, whereas the d-derivative does not.

Subsequent work by Fry and his colleagues at Parke-Davis confirmed the usefulness of the tetrafluorotyrosine moiety as a nonphosphorylatable analog, in this case for peptides that target the epidermal growth factor receptor (EGFR) (Fry et al. 1994). The phenylalanine-containing "parent" peptide acetyl-Leu-Ala-Glu-Glu-Ser-Ala-Phe-Glu-Glu displays a K_i of 150 μM, whereas the corresponding l- and d-tetrafluorotyrosine-containing derivatives exhibit relative inhibitory enhancements of threefold and eightfold, respectively. The Parke-Davis group also prepared peptides that contained other l-tyrosine analogs, including 3-fluorotyrosine, 3-iodotyrosine, and d-tyrosine, but all of these derivatives were ineffective EGFR inhibitors. Curiously, 3-iodotyrosine was subsequently found to serve as an excellent tyrosine replacement in a cyclic peptide targeting Src (Alfaro-Lopez et al. 1998).

Walsh, Cole, and their colleagues also examined the use of tetrafluorotyrosine as a tyrosine replacement in a C-terminal Src kinase (CSK)-targeted peptide (Cole et al. 1995; Kim and Cole 1998). However, in this case, the peptide serves as a substrate, rather than as an inhibitor, for CSK. These results suggest that the applicability of tetrafluorotyrosine as a nonphosphorylatable tyrosine replacement is kinase-dependent.

Lam and his collaborators have prepared a series of active site-directed peptides that target the Src tyrosine protein kinase (Lou et al. 1997). These

investigators employed both d- and l-napthylalanine (Nal) derivatives in place of the phosphorylatable tyrosine moiety in the sequence Gly-Ile-Tyr-Trp-His-His-Tyr. The corresponding phenylalanine derivative was not prepared; however, the d-Tyr was, which gives a measure of the inherent affinity of the peptide for Src. The IC_{50} for Gly-Ile-d-Tyr-Trp-His-His is 50 μM, which indicates that the peptide framework is, comparatively speaking, a remarkably effective peptide-based inhibitor. The corresponding Gly-Ile-Nal-Trp-His-His derivative exhibits only a twofold improvement in IC_{50} relative the d-Tyr analog. However, the doubly substituted Gly-Ile-Nal-Trp-His-His-Nal exhibits an IC_{50} of 4 μM, suggesting that the C-terminal Nal is able to access sites outside of the immediate active site region. Interestingly, one of the less effective inhibitors Gly-Ile-Nal-Trp-His-His-Tyr (IC_{50}=27 μM) proved to be remarkably selective for Src versus other closely related members of the Src kinase family (Lyn and Lck; IC_{50}>1 mM).

One of the difficulties associated with the acquisition of nonphosphorylatable tyrosine surrogates is their synthesis, which typically resorts to the use of achiral starting material. Following a resolution step, the analogs must then be appropriately protected for use in solid phase peptide synthesis. Some of these difficulties have been circumvented by Kim and Cole, who employed the enzyme tyrosine phenol lyase to prepare gram quantities of an assortment of fluorinated tyrosine analogs (Kim and Cole 1998). The Lawrence group has developed a library-driven strategy, which allows one to prepare and subsequently screen a wide assortment of commercially available aryl-containing amines as peptide-based nonphosphorylatable tyrosine analogs (Niu and Lawrence 1997a,b). In spite of the fact that these are peptide derivatives, issues related to synthesis, resolution, and protection of these tyrosine substitutes are all bypassed.

Although the most common protein kinase peptide substrates possess a phosphorylatable residue embedded within the interior of the peptide, protein kinases will also phosphorylate peptides containing tyrosine, serine, and threonine moieties appended off the N- or C-terminus of these substrates. For example, Src catalyzes the phosphorylation of Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu-Tyr-amide (the arginine residues are present for assay purposes, not enzyme recognition). C- and N-terminal residues can be readily appended onto the active site-directed peptide after solid phase peptide synthesis. This allows one to employ potential tyrosine analogs that are not protected, possess functionality that might not survive the harsh conditions of peptide synthesis, and even lack the standard α-stereocenter. The synthetic strategy utilizes a solid phase peptide synthesis support (Kaiser's oxime resin) that allows the tyrosine analog to be attached to the synthesized peptide in a fashion that simultaneously promotes cleavage from the resin (Kaiser et al. 1989). For example, a wide assortment of phenylethylamine derivatives was attached to the C-terminus of a Src active site-directed peptide (Niu and Lawrence 1997a,b).