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Invited Review

A historical overview of protein kinases and their targeted small molecule inhibitors

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ABSTRACT

Protein kinases play a predominant regulatory role in nearly every aspect of cell biology and they can modify the function of a protein in almost every conceivable way. Protein phosphorylation can increase or decrease enzyme activity and it can alter other biological activities such as transcription and translation. Moreover, some phosphorylation sites on a given protein are stimulatory while others are inhibitory. The human protein kinase gene family consists of 518 members along with 106 pseudogenes. Furthermore, about 50 of the 518 gene products lack important catalytic residues and are called protein pseudokinases. The non-catalytic allosteric interaction of protein kinases and pseudokinases with other proteins has added an important regulatory feature to the biochemistry and cell biology of the protein kinase superfamily. With rare exceptions, a divalent cation such as Mg^{2+} is required for the reaction. All protein kinases exist in a basal state and are activated only as necessary by divergent regulatory stimuli. The mechanisms for switching between dormant and active protein kinases can be intricate. Phosphorylase kinase was the first protein kinase to be characterized biochemically and the mechanism of its regulation led to the discovery of cAMP-dependent protein kinase (protein kinase A, or PKA), which catalyzes the phosphorylation and activation of phosphorylase kinase. This was the first protein kinase cascade or signaling module to be elucidated. The epidermal growth factor receptor-Ras-Raf-MEK-ERK signaling module contains protein-tyrosine, protein-serine/threonine, and dual specificity protein kinases. PKA has served as a prototype of this enzyme family and more is known about this enzyme than any other protein kinase. The inactive PKA holoenzyme consists of two regulatory and two catalytic subunits. After binding four molecules of cAMP, the holoenzyme dissociates into a regulatory subunit dimer (each monomer binds two cAMP) and two free and active catalytic subunits. PKA and all other protein kinase domains have a small amino-terminal lobe and large carboxyterminal lobe as determined by X-ray crystallography. The N-lobe and C-lobe form a cleft that serves as a docking site for MgATP. Nearly all active protein kinases contain a K/E/D/D signature sequence that plays important structural and catalytic roles. Protein kinases contain hydrophobic catalytic and regulatory spines and collateral shell residues that are required to assemble the active enzyme. There are two general kinds of conformational changes associated with most protein kinases. The first conformational change involves the formation of an intact regulatory spine to form an active enzyme. The second conformational change occurs in active kinases as they toggle between open and closed conformations during their catalytic cycles. Because mutations and dysregulation of protein kinases play causal roles in human disease, this family of enzymes has become one of the most important drug targets over the past two decades. Imatinib was approved by the United States FDA for the treatment of chronic myelogenous leukemia in 2001; this small molecule inhibits the BCR-Abl protein kinase oncoprotein that results from the formation of the Philadelphia chromosome. More than two dozen other orally effective mechanism-based small molecule protein kinase inhibitors have been subsequently approved by the FDA. These drugs bind to the ATP-binding site of their target enzymes and extend into nearby hydrophobic pockets. Most of these protein kinase inhibitors prolong survival in cancer patients only weeks or months longer than standard cytotoxic therapies. In contrast, the clinical effectiveness of imatinib against chronic myelogenous leukemia is vastly superior to that of

Abbreviations: AKAP, A-Kinase Anchoring Protein; ALL, acute lymphoblastic leukemia; A.S., activation segment; CDK, cyclin-dependent kinase; CML, chronic myelogenous leukemia; C-spine, catalytic spine; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; GIST, gastrointestinal stromal tumor; HΦ or Φ, hydrophobic; IGF-1R, insulin-like growth factor-1 receptor; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; Ph^+, Philadelphia chromosome positive; PKA, protein kinase A; pY, phosphotyrosine; R-spine, regulatory spine; Sh, shell; VEGFR, vascular endothelial growth factor receptor.

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any other targeted protein kinase inhibitor with overall survival lasting a decade or more. However, the near universal and expected development of drug resistance in the treatment of neoplastic disorders requires new approaches to solve this therapeutic challenge. Cancer is the predominant indication for these drugs, but disease targets are increasing. For example, we can expect the approval of new drugs inhibiting other protein kinases in the treatment of illnesses such as hypertension, Parkinson's disease, and autoimmune diseases.

Contents

1. The protein kinase enzyme family

Protein kinases play pivotal roles in nearly every aspect of cellular function [1]. They control metabolism, transcription, cell division and movement, programmed cell death, and they participate in the immune response and nervous system function. Protein phosphorylation involves the balanced action of protein kinases and phosphoprotein phosphatases making phosphorylation–dephosphorylation an overall reversible process [1,2]. Owing to the overall importance of protein phosphorylation, considerable effort has been expended to determine the assorted functions of protein kinase signal transduction pathways [1]. Moreover, dysregulation of protein kinases occurs in many diseases including cancer and inflammatory disorders.

Protein kinases can modify the function of a protein in almost every conceivable way. Protein phosphorylation can increase or decrease enzyme activity and it can alter other biological activities such as transcription and translation. Moreover, some phosphorylation sites on a given protein are stimulatory while others are inhibitory. Phosphorylation may stabilize or destabilize a

protein or promote movement of a protein from one cellular location to another. One of the mechanisms responsible for these various outcomes involves phosphorylation-induced changes in protein–protein interactions such as the binding of SH2 domains to protein-phosphotyrosine. The innumerable protein kinase substrates and the increases or decreases in biological activity that result from phosphorylation contribute to both the complexity and the nuances of signal transduction.

Manning et al. [1] reported that the human protein kinase gene family consists of 518 members along with 106 pseudogenes. The protein kinase family may be the second largest enzyme family and the fifth largest gene family in humans. Based upon a human gene number of 23,000, the protein kinase gene family follows C_2H_2 zinc finger proteins (3% of the total), G-protein coupled receptors (2.8%), the immunoglobulin/major histocompatibility complex protein family (2.8%), and the protease enzyme family (1.9%) [3,4]. Estimates of the total number of human genes has declined from 23,000 to 19,000 over the past dozen years [5]. Manning et al. [1] reported that chromosomal mapping revealed that 244 of 518 protein kinase genes map to disease loci or cancer amplicons (gene amplifications), a result that further emphasizes the importance of protein kinase inhibitors as potential drug targets.

Protein kinases are enzymes that catalyze the following reaction:

$$MgATP^{1-} + \text{protein–O : H} \rightarrow \text{protein–O : } PO_3{}^{2-} + MgADP + H^+$$

Note that the phosphoryl group ($PO_3{}^{2-}$) and not the phosphate ($OPO_3{}^{2-}$) group is transferred from ATP to the protein substrate. Numerous procedures for the measurement of protein kinase activities have been developed, but the "gold standard" involves the transfer of radiophosphate from [γ-^{32}P]ATP to a protein or peptide substrate using phosphocellulose P81 anion exchange paper to capture the phosphorylated product [6,7]. This method can be used with cell extracts or purified enzyme and can be performed manually or robotically. With purified enzymes, a non-radioactive assay was developed that involves the measurement of ADP formation by coupling its reaction with phosphoenolpyruvate to form pyruvate and ATP (the pyruvate kinase reaction) and monitoring the rate of conversion of pyruvate to lactate by following the decrease of NADH (the lactate dehydrogenase reaction) spectrophotometrically [8,9].

A divalent cation such as Mg^{2+} is required for the reaction. The activity of some protein kinases *in vitro* is greater with Mn^{2+} than Mg^{2+}, but the cellular concentration of Mg^{2+} is much greater than that of Mn^{2+} so that the predominant physiological substrate is $MgATP^{1-}$. Serine and threonine contain an alcoholic side chain while tyrosine contains a phenolic side chain. Based upon the nature of the phosphorylated –OH group (alcohol or phenol), these proteins are classified as protein-serine/threonine kinases (385 members), protein-tyrosine kinases (90 members), and tyrosine-kinase like proteins (43 members). Of the 90 protein-tyrosine kinases, 58 are receptor and 32 are non-receptor proteins [1].

2. Phosphorylated proteins

Casein, a milk protein, and phosvitin, an egg yolk protein, are two of the earliest known phosphoproteins [10]. Casein contains about 3% and phosvitin contains about 10% phosphorus by weight. The latter contains one phosphate group for every two amino acid residues thereby making it the most highly phosphorylated protein in nature. Lipmann and Levine identified phosphoserine in phosvitin in 1932 [10]. At the time threonine was unknown. W.C. Rose and two of his graduate students described it as a dietary essential amino acid in 1935 [11] and de Verdier identified phosphothreonine in casein in 1953 [12].

In 1979, Eckhart et al. [13] discovered that polyoma middle T antigen and large T antigen are phosphorylated. In terms of its electrophoretic and chromatographic mobility, the phosphorylated residue behaved identically to phosphotyrosine and differently from phosphorylated serine, threonine, lysine, or histidine. Hunter and Sefton [14] reported that the ratio of phosphoserine/phosphothreonine/phosphotyrosine in normal animal cell proteins was about 3100/260/1 (chicken cells in culture). However, more recent work suggests a ratio of 48/7/1 (HeLa Cells in culture) [15]. Despite the relative paucity of protein-phosphotyrosine residues, they play key roles in signal transduction and are essential for animal life.

3. Protein kinases, second messengers, and protein kinase cascades

3.1. Protein kinases and their activation by second messengers

Working in the Ben May Laboratory for Cancer Research at the University of Chicago, Williams-Ashman and Kennedy [16] reported that protein phosphorylation was especially active in malignant cells such as Ehrlich ascites tumor cells. Subsequently, Kennedy and Smith isolated radioactive phosphoserine of very high specific activity from the protein fraction of these tumor cells after incubation with [^{32}P]-phosphate [17]. They demonstrated that the phosphate moiety of phosphoserine in the protein fraction turns over rapidly. Although the physiological significance of this rapid turnover in normal and malignant tissues was unknown at the time, they wrote that such turnover "suggests a function of some importance" [17]. Their observations represented a harbinger of regulatory phosphorylation in signal transduction.

In 1954, Burnett and Kennedy [18] were the first to characterize protein kinase enzyme activity. They used a rat liver mitochondrial fraction as the source of their enzyme and fresh rat liver mitochondria to generate [γ-^{32}P]-ATP *in situ*. They found the γ-globulin, bovine serum albumin, lysozyme, and ovalbumin failed to serve as substrates whereas casein was readily phosphorylated. They isolated and identified [^{32}P]-phosphoserine following acid hydrolysis of the casein product. Furthermore, they demonstrated that chemically isolated [γ-^{32}P]-ATP serves as substrate and that Mg^{2+} was required for protein kinase activity. Their cell extracts most likely contained casein kinase-1, casein kinase-2, or both. Casein kinases occur in particulate cell fractions accounting for the presence of activity in the mitochondrial fraction. This was the only paper that these authors published on protein kinases leaving later work to other investigators. In pioneering work 26 years later (1980), Hunter and Sefton reported that the transforming gene product of Rous sarcoma virus (v-Src) catalyzes the phosphorylation of tyrosine, which represents the first study explicitly demonstrating protein-tyrosine kinase enzyme activity [14].

In 1955, Fischer and Krebs [19,20] and Sutherland and Wosilait [21] characterized the first specific protein kinase (phosphorylase kinase), which catalyzes the ATP-dependent phosphorylation of the less active phosphorylase *b* to produce the more active phosphorylase *a*. In 1958, Sutherland's group described the role of cAMP, the first of the second messengers, that leads to the activation of phosphorylase [22,23]. cAMP is a heat-stable compound that was characterized initially by Cook et al. [24]. The first messenger is a hormone (*e.g.*, epinephrine, glucagon) that leads to the activation of adenylyl cyclase and to the generation of cAMP [25]. This led to the discovery of protein kinase A (cyclic AMP-dependent protein kinase), a protein-serine/threonine kinase, by Walsh, Perkins, and Krebs in 1968 [26]. The quest for protein kinases activated by second messengers led to the discovery of protein kinase G (activated by cGMP) by Kuo and Greengard in 1970 [27] and protein

kinase C (activated by Ca^{2+}) by Inoue and Nishizuka et al. in 1977 [28]. Many more protein kinases were discovered in rapid succession using cDNA cloning methodologies. Hunter speculated in 1994 that there may be two thousand protein kinase genes [29]. This was at a time when educated guesses suggested that there would be ≈75,000 human genes. Both of these estimates were excessive by factors of three to four.

3.2. Protein kinase groups

In 2002, Manning et al. [1] published their landmark paper describing the protein kinase complement of the human genome (the kinome). The eukaryotic protein kinase (ePK) component consisted of 478 genes and 40 atypical genes (aPK) for a total of 518. They divided the eukaryotic protein kinase component into the following nine groups. (I) The AGC group consists of 63 members and contains the PKA, PKG, PKC families along with Akt1/2/3 (PKB1/2/3), Aur1/2/3 (Aurora kinase), PDK1 (phosphoinositide-dependent kinase), and RSK1/2/3/4 (ribosomal protein S6 kinase). (II) The CAMK group consists of 74 members and contains calcium/calmodulin-dependent protein kinases including CaMK1/2/4, PhKγ1/2 (phosphorylase kinase), MAPKAPK2/3/5 (mitogen-activated protein kinase activating protein kinases), Nek1–11 (Never in mitosis kinases), and MLCK (myosin light chain kinases). (III) The CK1 group consists of 12 members and contains the CK1α/γ/δ/ε (casein kinase 1), TTBK1/2 (tau tubulin kinase), and the VRK1/2/3 (vaccinia-related kinase) families. (IV) The CMGC group consists of 61 members and contains the CDK (cyclin-dependent protein kinases, CDK1–11), MAPK (ERK1–5), GSK3 (glycogen synthase kinase), and CDKL (CDK like, CDKL1–5) families. (V) The STE group (related to yeast non-mating or sterile genes) contains 47 members and consists of MAPK cascade families (Ste7/MAP2K, Ste11/MAP3K, and Ste20/MAP4K). MEK1/2/5/7 of the Ste7 family are dual specificity protein kinases that catalyze the phosphorylation of tyrosine and then threonine residues of the target ERK/MAP kinases. (VI) The TK (tyrosyl kinase) group consists of 90 members including 58 receptor (*e.g.*, EGFR, FGFR, Flt, InsulinR, PDGFR) and 32 non-receptor tyrosine kinases (*e.g.*, Abl, Eph, JAK, and Src). (VII) The TKL (tyrosyl kinase-like) group is a diverse family that resembles both protein-tyrosine and protein-serine/threonine kinases; it contains 43 members and consists of MLK1–4 (mixed-lineage kinases), LISK (LIMK/TESK for LIM (Lin-11, Isl-1, Mec-3) kinase and testes expressing serine kinase), IRAK (interleukin-1 receptor-associated kinase), Raf, RIPK (receptor-interacting protein kinase, or RIP), and STRK (activin and transforming growth factor receptors). LIM kinase contains paired zinc fingers that participate in protein–protein interactions as opposed to DNA binding [30]. (VIII) The RCG (receptor guanylyl cyclase) group contains 5 members and is similar in domain sequences to protein-tyrosine kinases. (IX) The final group, labeled OTHER, is diverse with 83 members.

Besides these nine protein kinase groups, there were an additional 40 enzymes that were classified as atypical. Pyruvate dehydrogenase kinase, which occurs within the mitochondrion, is one of these atypical kinases. Linn et al. [31] reported that pyruvate dehydrogenase was inhibited following its phosphorylation; this inhibition was reversed by the action of mitochondrial protein phosphatases. At the time of this work (1969), regulation of biological activities by phosphorylation was thought to be an esoteric type of control system restricted to glycogen metabolism. Phosphorylase kinase and phosphorylase were activated by phosphorylation [32] while glycogen synthase was inhibited following its phosphorylation [33]. Edwin Krebs wrote that "it was not until 1969, with the finding from Lester Reed's laboratory that pyruvate dehydrogenase is regulated by phosphorylation-dephosphorylation, that the field broke out of the more restricted area" [32]. This was the bellwether for the description of the multitudinous processes that are regulated by protein phosphorylation.

The initial studies on the receptor protein-tyrosine kinase group were performed by Cohen [34]. He described epidermal growth factor (EGF), its receptor (EGFR), and its biochemical actions. Cohen's group reported that EGF stimulated protein phosphorylation in A431 cell membranes [35]. A431 cells, which overexpress EGFR and are widely used in EGFR studies, were originally derived from a vulvar carcinoma obtained from an 85-year old female [36]. Initially, Carpenter et al. [37] thought that phosphothreonine was generated following EGF treatment. Using a procedure that obviated the comigration of phosphothreonine and phosphotyrosine, Ushiro and Cohen found instead that EGF stimulated tyrosine phosphorylation [38]. Using purified EGFR with an apparent molecular weight of 170 kDa, Chinkers and Cohen demonstrated that EGF stimulated tyrosine phosphorylation [39]. Furthermore, Cohen et al. [40] found that the solubilized 170 kDa polypeptide contains both EGF binding and protein kinase activities.

Downward et al. [41] and Lin et al. [42] cloned and sequenced EGFR cDNA, and confirmed that the receptor contains a protein kinase domain. Downward et al. [41] hypothesized that EGFR consists of an extracellular domain (621 residues), a transmembrane segment (23 predominantly hydrophobic residues), and an intracellular domain (542 residues). Subsequently, all receptor protein-tyrosine kinases were found to have a similar overall architecture. These studies demonstrated that the EGF receptor was a protein-tyrosine kinase, the first of the receptor protein-tyrosine kinases, and this was a revolutionary finding at the time (see Ref. [43] for a historical review). EGFR was also the first receptor that provided evidence for a relationship between receptor overexpression and cancer [44]. EGFR is among the most studied receptor protein-tyrosine kinases owing to its general role in signal transduction and in the oncogenesis of breast, colorectal, lung, pancreatic, and other cancers [45].

All protein kinases exist in a basal state and are activated only as necessary by divergent regulatory stimuli [46]. Ligand-induced dimerization and activation segment phosphorylation are required for the activation of most receptor protein-tyrosine kinases with the exception of EGFR where such phosphorylation is not required for activation [45]. ERK1/2 are activated following activation segment tyrosyl and threonyl residue phosphorylation as catalyzed by MEK1/2 [47,48]. Src family kinase activation is initiated by dephosphorylation of an inhibitory phosphotyrosine followed by activation segment phosphorylation [49,50]. CDKs are activated by their cognate cyclins and calcium/calmodulin-dependent protein kinases (CaM kinases) are activated by calcium-calmodulin complexes [51–53]. Thus, the specific mechanisms underlying protein kinase activation and deactivation are diverse and kinase specific.

The mechanisms for the interconversion of dormant and active protein kinases are often intricate. Taylor et al. [46] refer to this interconversion as switching. Protein kinases have not evolved to continuously catalyze the phosphorylation of thousands of molecules per minute like hexokinase (k_{cat} = 6000 min^{-1}), a general metabolic enzyme [54]. For example, when a receptor protein-tyrosine kinase is activated by its ligand, the chief phosphorylated product is the receptor itself mediated by the autophosphorylation of one receptor protein kinase by another. This observation is in agreement with the low ratio of phosphotyrosine in proteins when compared with phosphoserine/threonine as noted previously [15]. Thus, classical Michaelis–Menten steady-state enzyme kinetics, which is based on the premise that the substrate concentration exceeds that of the enzyme by several orders of magnitude, fails to apply to the physiological function of protein kinases when the concentrations of the enzyme and substrate are similar.

3.3. Regulation of protein kinase A activity

The regulation of PKA by cAMP, its cognate second messenger, is unique in that it does not involve activation segment phosphorylation–dephosphorylation, but involves cAMP binding to the regulatory subunits of a protein kinase holoenzyme [46]. In March 1970, Gill and Garren reported their resolution of the bovine adrenal cortical PKA holoenzyme into a cAMP-binding fraction and a protein kinase catalytic fraction [55]. They proposed that the cAMP-binding fraction suppresses protein kinase catalytic activity and binding of cAMP to its receptor relieves this suppression. In early 1969, Tao and Salas in Fritz Lipmann's laboratory were able to resolve rabbit reticulocyte PKA holoenzyme into regulatory and catalytic subunits by sucrose density gradient centrifugation. Although Lipmann was known for his astute scientific intuition [56], he was skeptical of these results and refused to have them published. After the appearance of the Gill and Garren paper, he changed his mind overnight and submitted a paper that reported these findings, which was published in June 1970 [57]. Lipmann, the discoverer of phosphoserine in phosvitin [10], soon thereafter left the protein kinase field because he thought that it had become too crowded. He and many others did not realize the widespread nature of regulatory protein phosphorylation, a field that is still expanding 45 years later.

Protein kinase A consists of two general types (I and II). There are four non-redundant R-subunits (RIα, RIβ, RIIα, and RIIβ) in humans and mice, which differ in their patterns of expression and cellular location, and three catalytic subunits (Cα/β/γ) in humans [58] and two in mice (Cα/β) [59]. The inactive PKA holoenzyme consists of two regulatory and two catalytic subunits. After binding four molecules of cAMP, the holoenzyme dissociates into a regulatory subunit dimer (each monomer binds two cAMP) and two free active catalytic subunits according to the following chemical equation [60]:R_2C_2+ 4 cAMP ⇔ $R_2(cAMP)_4$ + 2 C

$$R_2C_2 + 4\,cAMP \Leftrightarrow R_2(cAMP)_4 + 2\ C$$

The RI subunits possess a pseudosubstrate binding site while the RII subunits are substrates as well as inhibitors of the C subunit. However, the phosphorylated RII-dimer does not dissociate from the catalytic subunits in the absence of cAMP [58]. The R subunits are tightly bound to the C subunits thereby blocking C subunit interaction with external protein substrates.

3.4. Signal transduction cascades

The PKA-phosphorylase kinase-phosphorylase pathway represents the first known signal-transduction cascade, which consists of a series of two protein-serine kinases (Fig. 1A). This pathway is initiated by a hormone or neurotransmitter, the first messenger, which leads to the activation of adenylyl cyclase and the generation of cAMP, the second messenger. cAMP activates PKA, which then leads to the activation of phosphorylase kinase. The latter enzyme then catalyzes the phosphorylation and activation of glycogen phosphorylase. The latter enzyme catalyzes the phosphorolysis (cleavage by P_i) of glycogen leading to the formation of glucose 1-phosphate. If the first messenger were a catecholamine such as epinephrine as part of the flight or fright response, the subsequent production and metabolism of glucose 1-phosphate supports this response by generating energy in the form of ATP.

A "mixed" signal transduction cascade includes a protein-tyrosine kinase and a protein-serine/threonine kinase in series [32]. For example, the EGFR cascade consists of one protein-tyrosine kinase (EGFR), two protein-serine/threonine kinases (B-Raf and ERK1), and a dual-specificity protein kinase (MEK1) along with auxiliary protein components (Fig. 1B). The activation of the

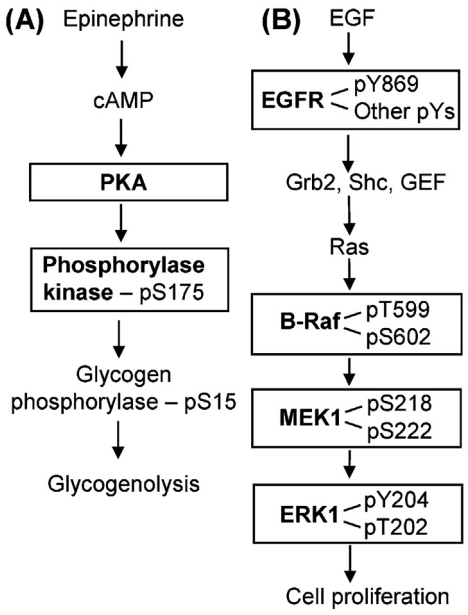

Fig. 1. (A) The protein kinase A-phosphorylase kinase signal transduction cascade. (B) The EGFR mixed protein kinase cascade with protein-tyrosine, protein-serine/threonine, and dual specificity protein kinase participants. Residue numbers correspond to human proteins.

EGFR receptor and autophosphorylation of selected endogenous tyrosines leads to the recruitment of Grb2, Shc, and GEF (guanine nucleotide exchange factor) and the subsequent conversion of inactive Ras-GDP to active Ras-GTP [45]. Active Ras-GTP promotes the activation of the Raf-MEK-ERK protein kinase cascade leading to cell proliferation [47,48,61]. The activation of the Raf family of protein-serine/threonine kinases (A-, B-, and C-Raf) occurs by an intricate multistage process. The Raf kinases have restricted substrate specificity and catalyze the phosphorylation and activation of MEK1 and MEK2. The latter are ubiquitous dual-specificity non-receptor protein kinases that mediate the phosphorylation of tyrosine and then threonine in ERK1 or ERK2, their only known physiological substrates.

ERK1 and ERK2 contain Thr-Glu-Tyr within their activation segments. Anderson et al. [62] hypothesized (≈1990) that the dual phosphorylation of tyrosine and threonine resulted from the action of two different protein kinases: a protein-threonine and a protein-tyrosine kinase. Now we know that MEK1 or MEK2 is able to catalyze the phosphorylation of tyrosine and then threonine in ERK1/2. MEK1/2 do not catalyze the phosphorylation of denatured ERK1/2 or ERK1/2 peptides indicating that the overall protein conformation of ERK1/2 is important for recognition by MEK1/2 [63]. This phosphorylation activates ERK1/2, which are protein-serine/threonine kinases. Unlike the Raf kinases and MEK1/2, which have narrow substrate specificity, ERK1 and ERK2 are broad specificity protein kinases that have dozens of cytosolic and nuclear substrates including components that lead to cell proliferation. PKA and PKC are also a broad specificity protein kinases.

One hypothetical consequence of a signaling cascade is that of amplification. One protein kinase can potentially catalyze the phosphorylation of thousands of substrate molecules. If the substrate is a protein kinase, it too can catalyze the phosphorylation of thousands of substrate molecules, *etc.*, thereby leading to amplifications exceeding 1×10^6. Moreover, such regulatory amplifications can occur on a millisecond time scale [64]. The original definition of a cascade is a series of waterfalls. The amount of water that goes over the last waterfall is the same as that going over the first, and amplification in a waterfall cascade does not occur. As noted next,

Table 1
Concentrations of Ras, Raf, MEK, and ERK in HeLa cells following EGF stimulation.[a]

	Total (nM)	Activated (nM)
Ras	400	200
Raf	13	6.5
MEK	1400	700
ERK	960	530

[a] Data from Ref. [65].

some amplification can occur in physiological signal-transduction cascades.

Fujioka et al. [65] measured the levels of Ras, Raf, MEK, and ERK in human HeLa cells (Table 1). They reported that the Ras content is about 30 times that of Raf. They found that the concentration of MEK is about 110 times that of Raf, but the concentration of ERK is only 69% that of MEK. Thus in this MAP kinase signaling module, the possibility of a 110-fold amplification from Raf to MEK exists. In contrast, this degree of amplification from MEK to ERK is unlikely. Fujioka et al. reported that EGF stimulation of HeLa cells leads to the activation of 50% of Ras, Raf, and MEK. This observation indicates that an approximate 100-fold amplification from Raf to MEK occurred in response to EGF. About 2/3rds of ERK was found in its activated state, but the actual concentration is less than that observed for MEK. These findings indicate that significant amplification from Raf to ERK occurred, but not a hypothetical increase amounting to several orders of magnitude. For protein kinases in general, the concentration of the kinase and the kinase substrates are usually within one or two orders of magnitude and overall pathway amplification is not as great as first imagined in the 1970s.

4. Structures of protein kinases

4.1. Primary structures of protein kinases

Czernilofsky et al. [66,67] reported the amino acid sequence of the Schmidt-Rupin strain of v-Src in 1980 based upon its nucleotide sequence and Shoji et al. [68] reported the sequence of the catalytic subunit of bovine PKA in 1981 using Edman degradation of cyanogen bromide and trypsin peptides. Owing to the incomplete nature of the protein sequence data bases at the time, the identification of v-Src as a protein kinase was not made until 1982 [69]. The signatures that enabled this association were the G-rich loop and sequence similarity near v-Src lysine 295 and PKA lysine 71, the latter of which Zoller and Taylor identified as the residue that reacts with *p*-fluorosulfonylbenzoyl-5′-adenosine, an ATP analog [70]. Thus, the first known primary structure of any protein kinase was that of v-Src, but at the time of its publication it was not known to be a protein kinase.

In a landmark paper published in 1988, Hanks et al. [71] analyzed the sequences of some five dozen protein-serine/threonine and protein-tyrosine kinases and divided the primary structures into 11 domains (I–XI). The catalytic domains contain 250–300 amino acid residues. Domain I is G-rich and contains the GxGxxG signature, which was later found to overlay bound ATP. Domain II contains Ala-Xxx-Lys and domain III contains a conserved glutamate residue that form a salt bridge with the Lys of Ala-Xxx-Lys in the active conformation. Domain IV contains a variable sequence with a conserved hydrophobic residue (Leu, Ile, or Val) and domain V consists of another variable sequence that was later found to form the αE-helix. Domain VIa contains a small residue such as glycine, two spacer residues, and two hydrophobic residues such as tyrosine/leucine and domain VIb contains a conserved Y/HRD sequence, which forms part of the catalytic loop. Domain VII contains a DFG signature and domain VIII contains a conserved APE sequence, which together represent the beginning and end of the protein kinase activation segment. Domain IX contains a conserved aspartate separated from a conserved glycine and domain X consists of a variable sequence that was later found to form the αG-helix. Domain XI contains a conserved hydrophobic residue separated from a downstream arginine, the latter of which forms a salt bridge with the E of APE of domain VIII. The elucidation of the X-ray structure of PKA provided an initial framework for understanding the role of the various domains on protein kinase function, which is considered next.

4.2. Secondary and tertiary structures of protein kinases

In pioneering studies reported in 1991, Knighton et al. described the X-ray crystal structure of the catalytic subunit of PKA bound to a heat-stable protein kinase inhibitor that mimics a peptide substrate (PDB: ID 2CPK) [72,73]. Protein kinase A and all other protein kinase domains have a small amino-terminal lobe and large carboxyterminal lobe [46]. The N-lobe and C-lobe form a cleft that serves as a docking site for ATP beneath the G-rich loop (Fig. 2A). Moreover, the structure revealed the location of the protein kinase signatures described by Hanks et al. [71] (Fig. 2B). At the time the primary structures of about 65 protein kinases were known. Based upon the nature of the conserved residues and their location in the protein kinase A structure, Knighton et al. [72] hypothesized that the bilobed structure would occur in all serine/threonine and tyrosine protein kinases and this prediction has proven correct [46]. Of the many hundreds of protein kinase domain structures in the protein data bank, all of them have the fundamental protein kinase fold first described for PKA [74].

Although the small N- and large C-lobes contribute to nucleotide binding, most of the interaction involves the N-lobe. The small lobe of protein kinases contains a conserved glycine-rich (GxGxΦG) ATP-phosphate-binding loop, which is the most flexible part of the lobe, where Φ refers to a hydrophobic residue. The glycine-rich loop of PKA consists of ^{50}GTGSFG^{55}. The tertiary structure shows that the penultimate phenylalanine (F54) and third glycine (G55) of the glycine-rich loop of PKA, shown in green, anchor the β-phosphate of ATP (Fig. 2C). The –NH and the hydroxyl groups of Ser53 of the G-rich loop interact with the γ-phosphate. The third glycine (G52) forms a hydrogen bond with G55. Lys72 of the β3-strand holds the α- and β-phosphates in position. Glu91 of the αC-helix forms a salt bridge with Lys72 and serves to stabilize its interactions with these phosphates.

The 6-amino group of the adenine base of ATP forms a hydrogen bond with the carbonyl oxygen of Glu121, the first hinge residue of PKA. The N-1 and N-7 of the adenine ring form a hydrogen bond with the main chain –NH group of the Val123 hinge residue and the –OH group of Thr183 near the beginning of the activation segment, respectively (Fig. 2C). Thr183 is unique to PKA and is not conserved among the protein kinase family. The adenine base interacts with several hydrophobic residues in the ATP-binding pocket including Leu49, Val57, Ala70, Met120, Tyr122, Val123 from the N-lobe and Leu173 from the C-lobe (not shown) [75]. As noted above, the β3-strand of all protein kinases typically contains an Ala-Xxx-Lys sequence, the lysine of which in PKA (K72) forms a salt bridge with a conserved glutamate near the center of the αC-helix (E91) (Fig. 2A). The presence of a salt bridge between the β3-lysine and the αC-glutamate is a prerequisite for the formation of active protein kinases. This salt bridge is very important and will be mentioned several times.

The C-lobe makes a major contribution to protein/peptide substrate binding, but it also participates in nucleotide binding. Asp184 (the D of DFG) binds to Mg^{2+}(1), which in turn coordinates with the β- and γ-phosphates of ATP (Fig. 2C). The γ-phosphate interacts with Lys168 of the catalytic loop, a residue that is conserved within the protein-serine/threonine kinase family. The

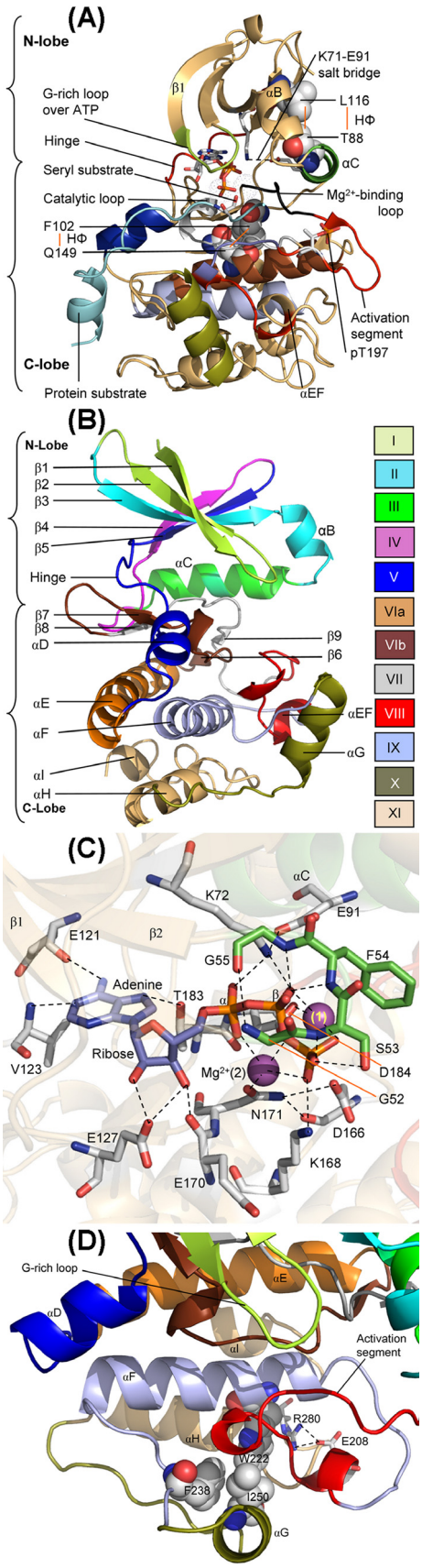

Fig. 2. Structure of the catalytic subunit of PKA. (A) ATP occurs in the cleft between the small and large lobes under the G-rich loop. The peptide substrate is colored cyan. (B) PKA secondary structure with color-coded Hanks domains. (C) ATP binding pocket. (D) Hydrophobic interactions around the αGHI loop. Dashed lines represent polar contacts. Adapted from PDB ID: 4DH3. Figs. 2–7 were prepared using the PyMOL Molecular Graphics System Version 1.5.0.4 Schrödinger, LLC.

2′ and 3′-hydroxyl groups of the ATP ribose hydrogen bond with Glu127 (the first residue following the hinge) and the 3′-hydroxyl binds to Glu170 of the catalytic loop. Leu173 is the only C-lobe residue that makes hydrophobic contacts with ATP (not shown). Most small molecule protein kinase inhibitors make contact with many of the residues of the ATP-binding pocket that are homologous to the residues mentioned in this section.

The C-lobe of protein kinases contains a mobile activation segment with an extended conformation in active enzymes and a closed conformation in dormant enzymes. The first residues of the activation segment of protein kinases consist of DFG (Asp-Phe-Gly). In the active state of PKA and other protein kinases, the aspartate side chain (D184 of PKA) faces into the ATP-binding pocket and coordinates Mg^{2+}(1). The Mg^{2+}-binding loop (Fig. 2A) of PKA consists of the first five residues of the activation segment (^{184}DFGFA^{188}).

In protein-serine/threonine kinases, the phosphorylatable serine or threonine of the protein substrate interacts with backbone residues near the end of the activation segment. These correspond to ^{199}CGTP^{202} of PKA, which make up the protein-substrate positioning segment. In contrast to protein-serine/threonine kinases, the R-group of a proline residue in the corresponding segment of protein-tyrosine kinases serves as a platform that interacts with the tyrosyl residue of the peptide/protein substrate that is phosphorylated [76].

The activation segment of protein kinases contains a phosphorylatable residue and its phosphorylation is usually required for enzyme activation [46]. Because phosphorylation of PKA at residue Thr197 of the activation segment occurs during its biosynthesis, the kinase domain structure is that of an active kinase subject to inhibition by the regulatory subunit. Most other protein kinases exist in active and inactive conformations as described later for the Abl protein-tyrosine kinase. The phosphorylation of an activation segment residue is not required for the activation of some protein kinases including the EGFR, ErbB2, and ErbB4 protein-tyrosine kinases [45,77]. The activation segments of protein kinases including PKA typically ends with APE (Ala-Pro-Glu). The glutamate residue at the end of the activation segment of all protein kinases forms a salt bridge with an arginine residue in the αHI loop; this salt bridge consists of Glu208 and Arg280 in PKA (Fig. 2D).

Three conserved hydrophobic interactions in protein kinases contribute to enzyme stability. A hydrophobic contact between Thr88 of PKA, which is three residues N-terminal to the Glu91 in the αC-helix, with Leu116 near the N-terminus of the β4-strand helps to stabilize the small lobe. Another hydrophobic interaction involving Phe102 in the αC-β4 loop of the small lobe and Gln149 near the carboxyterminal end of the αE-helix in the large lobe further stabilizes the kinase domain (Fig. 2A). The GHI subdomain and the activation segment are bound to each other and to the αF-helix by a set of conserved hydrophobic interactions that involve Trp222 from the αF-helix, Ile250 from the αG-helix, and Phe238 in the αFG loop (Fig. 2D). Similar hydrophobic interactions occur within the protein kinase enzyme family.

4.3. The K/E/D/D signature motif

Nearly all active protein kinases contain a K/E/D/D (Lys/Glu/Asp/Asp) signature motif that plays important structural and catalytic roles. As noted above, the β3-strand typically contains an Ala-Xxx-Lys sequence, the lysine of which in PKA (K72) forms a salt bridge with a conserved glutamate near the center of the αC-helix (E91) of protein kinases. The presence of this salt bridge is a prerequisite for the formation of the active state and corresponds to the αC-inward conformation (Fig. 2A). By contrast, the β3-Lys and the αC-Glu of the dormant form of many, but not all, protein kinases fail to make contact and this structure

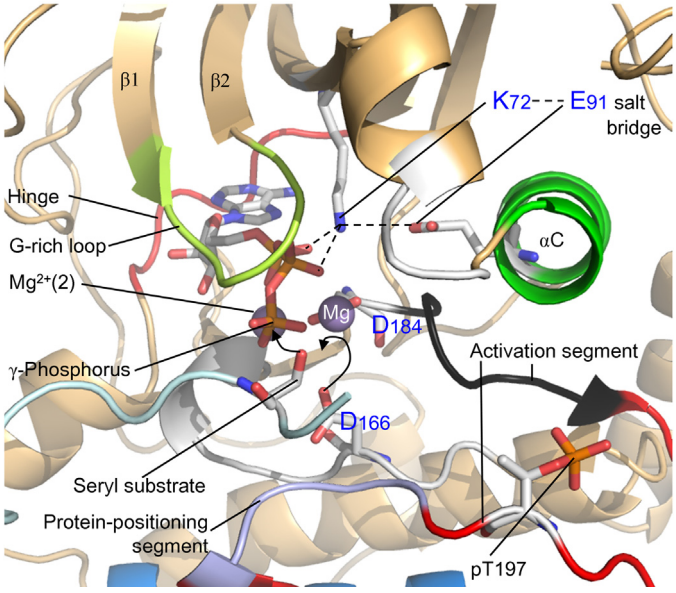

Fig. 3. Mechanism of the PKA reaction. The oxygen of the protein-serine substrate attacks the γ-phosphorus of ATP. Asp166 serves as a catalytic base by removing the proton from the seryl hydroxyl group.
Adapted from PDB ID: 1ATP.

corresponds to the displaced αC-outward conformation. The αC-inward conformation is necessary, but not sufficient, for the expression of full protein kinase activity. Lys72 of PKA also forms salt bridges with both the α- and β-phosphates of ATP (Fig. 2C).

The two aspartate residues in the K/E/D/D signature motif occur in the large lobe (Table 2). In PKA, Asp166 functions as a catalytic base that abstracts a proton from the protein-serine/threonine substrate residue thereby facilitating its in-line nucleophilic attack onto the γ-phosphorus atom of MgATP (Fig. 3) [78]. This base occurs within the catalytic loop of protein-serine/threonine kinases with a canonical H/YRDKPEN sequence [71]. In non-receptor protein-tyrosine kinases the catalytic loop has a canonical HRDLRAAN sequence while in receptor protein-tyrosine kinases the sequence is HRDLAARN. Asp184 is the first residue of the PKA activation segment found in the large lobe (the second D of K/E/D/D) and binds one of two essential Mg^{2+} ions.

Zheng et al. [75] determined the structure of the catalytic subunit of murine PKA bound to Mg^{2+}, ATP, and a peptide inhibitor that mimics a protein substrate. They prepared crystals under conditions of low and high [Mg^{2+}]. They reported that MgATP is found in a cleft between the small and large lobes. At low [Mg^{2+}], a single Mg^{2+} is bound to the aspartate of the DGF sequence and to the β- and γ-phosphates of ATP, which they labeled magnesium ion 1: Mg^{2+} (1). At high [Mg^{2+}], a second Mg^{2+} is bound to the Asn171 amide nitrogen within the catalytic loop and the α- and γ-phosphates of ATP, which they labeled Mg^{2+}(2) (Fig. 2C).

Bastidas et al. [79] determined the structure of the catalytic subunit of murine PKA linked to Mg^{2+}, a peptide substrate, and an ATP analog (AMP-PNP, 5′-adenylyl-β, γ-imidodiphosphate). The latter is an ATP congener that ordinarily fails to react. However, Bastidas et al. found that this ATP analog transfers its terminal phosphoryl group to the peptide substrate. They studied crystals that contained 55% intact AMP-PNP and an unphosphorylated peptide substrate and 45% displaying AMP-PN and a phosphorylated peptide substrate. Their results implicated Mg^{2+}(2) as the more stably bound ion. After the transferase reaction, they found that Mg^{2+}(2) recruits a water molecule thereby retaining its octahedral coordination geometry and it remains in the active site while Mg^{2+}(1) is released. This finding on the order of release of Mg^{2+}(2) and Mg^{2+}(1) is in contrast with earlier studies [80]. Bastidas et al. [79] hypothesize that the mechanism of all protein kinases will require two magnesium ions for catalysis. The catalytic-loop asparagine and activation-segment aspartate each interact with a Mg^{2+} ion and they play crucial roles in the transferase reaction.

5. Structure of the protein kinase A hydrophobic skeleton

5.1. The regulatory spine

Kornev et al. [81,82] compared the spatial arrangements of amino acid residues in about two dozen active and inactive protein kinases using a local spatial pattern (LSP) alignment algorithm. They used this information to establish the existence of a regulatory and a catalytic spine within the protein kinase domain. In contrast to protein kinase amino acid signatures such as Y/HRD or DFG, the residues that constitute the spines were not identified by sequence analyses *per se*. Rather, the spines were identified by their three-dimensional location as determined from their X-ray crystal structures.

The local spatial pattern alignment analysis revealed a skeleton of four nonconsecutive hydrophobic residues that constitute a regulatory or R-spine and eight hydrophobic residues that constitute a catalytic or C-spine (Fig. 4A and B). The R-spine interacts with a conserved aspartate (D220) in the αF-helix. As noted later in this section, there are three conserved "shell" residues that interact with the R-spine. Altogether each protein kinase contains 16 amino acids that make up this protein kinase skeletal assembly. Each spine consists of residues derived from both the small and large lobes. The regulatory spine contains residues from the activation segment and the αC-helix, whose conformations are important in defining active and inactive states. The catalytic spine facilitates ATP binding while the regulatory spine dictates the positioning of the protein substrate so that catalysis occurs. The correct alignment of R-spines is necessary for the fabrication of active kinases.

The PKA regulatory spine consists of a residue from the beginning of the β4-strand (Leu106), from the C-terminal end of the αC-helix (Leu95), the phenylalanine of the activation segment DFG (Phe185), along with the H/YRD-tyrosine (Y164) of the catalytic loop (Fig. 4B). Leu95 and comparable amino acids from other protein kinases are four residues C-terminal to the conserved αC-glutamate. The backbone of Tyr164 is anchored to the αF-helix by a hydrogen bond to a conserved aspartate residue (Asp220). The protein-substrate positioning segment, the activation segment, the αHI-loop, and the catalytic spine of protein kinase domains form hydrophobic contacts with the αF-helix [81].

5.2. The catalytic spine

The catalytic spine of protein kinases consists of residues from the small and large lobes and is completed by the adenine base of ATP [82]. The two residues of the small lobe of PKA that bind to the adenine group of ATP include Val57 from the beginning of the β2-strand and Ala70 from the conserved Ala-Xxx-Lys of the β3-strand. Furthermore, Leu173 from the middle of the large lobe β7-strand binds to the adenine base in the active enzyme. Leu172 and Ile174, hydrophobic residues that flank Leu173, bind to Met128 at the beginning of the αD-helix. The αD-helix Met128 binds to Leu227 and Met231 in the αF-helix (Fig. 4B). Note that both the R-spine and C-spine are anchored to the αF-helix (Fig. 4A), which is a very hydrophobic component of the enzyme that traverses the entire large lobe and is not exposed to the solvent. The αF-helix serves as a sacrum that supports the spines, which in turn anchor the protein kinase catalytic muscle. Table 3 lists the residues of the spines of the catalytic subunit of murine PKA.

Table 2
Important residues in murine PKA and human Abl.[a]

	PKA	Abl	Inferred function	Hanks no.
N-lobe				
Glycine-rich loop; GxGxΦG	^{50}GTGSFG^{55}	^{249}GGGQYG^{254}	Anchors ATP β- and γ-phosphates	I
β3-Lys (K of K/E/D/D)	72	271	Anchors ATP α- and β-phosphates	II
αC-Glu (E of K/E/D/D)	91	286	Forms ion pair with β3-Lys	III
αC-β5-strand HΦ interaction	T88-L116	F283-I313	Stabilizes N-lobe	III–V
αC-β4 loop and αE helix HΦ contact	F102-Q149	H295-A350	Stabilizes N-lobe C-lobe interaction	IV–VI
Hinge residues	^{121}EYVAGG^{126}	^{316}EFMTYG^{321}	Connect N- and C-lobes	V
C-lobe				
αE-A.S. loop and A.S. HΦ-interaction	L162-R190	F359-L387	Stabilizes A.S.	VIb–VII
Catalytic loop Y/HRD (first D of K/E/D/D)	166	363	Catalytic base (abstracts proton)	VIb
Intracatalytic loop salt bridge	D166-K168	None	Stabilizes catalytic loop	VIb
Catalytic loop-A.S. H-bond	R165-F187	R362-L384	Stabilizes A.S.	VIb–VII
Catalytic loop-A.S. salt bridge	R165-pT197	Unknown	Stabilizes A.S.	VIb–VIII
Intracatalytic loop H-bonds	Y164-D166; Y164-N171; D166-N171	H361-D363 D363-N369	Stabilizes catalytic loop	VIb
Catalytic loop asparagine (N)	171	368	Chelates Mg^{2+}(2)	VIb
Activation segment	184–208	381–409		VII–VIII
A.S. DFG (second D of K/E/D/D)	184	381	Chelates Mg^{2+}(1)	VII
Mg^{2+}-positioning loop	^{184}DFGFA^{188}	^{381}DFGLS^{385}	Positions Mg^{2+}	VII
A.S. phosphorylation site	T197	Y393	Stabilizes A.S. after phosphorylation	VIII
Protein substrate-positioning loop	^{199}CGTP^{202}	^{400}KFPI^{403}	Constrains protein substrate	VIII
APE; end of the A.S.	206–208	407–409		VIII
APE and αH-αI loop salt bridge	E208-R280	E409-R483	Stabilizes A.S.	VIII–XI
UniProt KB ID	P68181	P00519		

[a] A.S., activation segment.

5.3. Shell residues stabilizing the R-spine

Going from the aspartate in the αF-helix up to the top residue in the β4-strand, Meharena et al. [74] labeled the regulatory spine residues RS0, RS1, RS2, RS3, and RS4 (Table 3). Using site-directed mutagenesis, these investigators identified three residues in murine PKA that stabilize the R-spine that they labeled Sh1, Sh2, and Sh3, where Sh refers to shell. Sh1 interacts with RS3 and Sh2 while Sh3 interacts with RS4. Sh2 is the classical gatekeeper residue, which interacts with Sh1 below it and with RS4 next to it (Fig. 4C). The name gatekeeper indicates the role of this residue in controlling access to hydrophobic pocket II adjacent to the adenine binding site [83,84] that is occupied by components of many small molecule inhibitors as described later. Based upon the local spatial pattern alignment data [81], only three of 14 amino acid residues in PKA surrounding RS3 and RS4 are conserved. These shell residues serve as collateral ligaments that stabilize the protein kinase regulatory spine [74]. The Sh1 Val104Gly mutant exhibited 5% of the catalytic activity of wild type PKA while the Sh2/Sh3 (Met120Gly/Met118Gly) double mutant was kinase dead. These results argue for the importance of shell residues in stabilizing the regulatory spine and contributing to an active protein kinase conformation.

6. The protein kinase A holoenzyme: R_2C_2

6.1. PKA signaling

As noted previously, the PKA holoenzyme consists of two general types (I and II) originally based upon their order of elution from DEAE-cellulose anion exchange resin; the type I enzyme elutes first at a lower salt concentration than the type II enzyme. As noted previously, PKA holoenzymes exist as an inactive tetrameric complex composed of two catalytic and two regulatory subunits (R_2C_2) [60]. Mammals possess four non-redundant R-subunits (RIα, RIβ, RIIα, and RIIβ), which differ in their patterns of expression and cellular location, and humans possess three catalytic subunits (Cα/β/γ) [58] while mice possess two (Cα/β) [59]. In general, the α-catalytic and α-regulatory subunits are expressed in all mouse

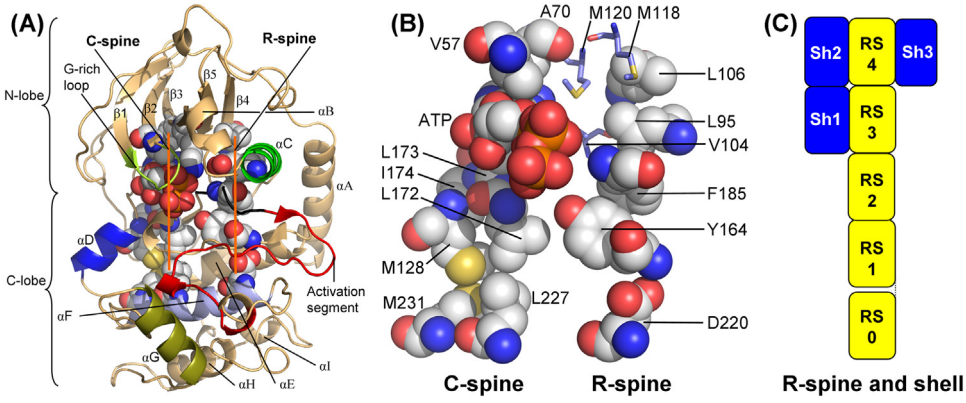

Fig. 4. The regulatory and catalytic spines of PKA. (A) The spines traverse the small and large lobes and are supported by the αF helix. The αA and αB helicies are unique to PKA and are not conserved in the protein kinase enzyme family. (B) Identity of the residues that constitute the C- and R-spines. (C) Interaction of the shell residues with the R-spine. (A) and (B) prepared from PDB ID: 1ATP.

Table 3
Murine PKA and human Abl R-spine, R-shell, and C-spine residues.

	Symbol	PKA[a]	Abl
Regulatory spine			
β4-strand (N-lobe)	RS4	Leu106	Leu301
αC-helix (N-lobe)	RS3	Leu95	Met290
Activation loop F of DFG (C-lobe)	RS2	Phe185	Phe382
Catalytic loop Tyr or His (C-lobe)[b]	RS1	Tyr164	His361
αF-helix (C-lobe)	RS0	Asp220	Asp421
R-shell			
Two residues upstream from the gatekeeper	Sh3	M118	Ile313
Gatekeeper; end of β5-strand	Sh2	M120	Thr315
αC-β4 loop	Sh1	V104	Val299
Catalytic spine			
β2-strand (N-lobe)		Val57	Val256
β3-AxK motif (N-lobe)		Ala70	Ala269
β7-strand (C-lobe)		Leu173	Leu370
β7-strand (C-lobe)		Leu172	Cys369
β7-strand (C-lobe)		Ile174	Val371
αD-helix (C-lobe)		Met128	Leu323
αF-helix (C-lobe)		Leu227	Leu428
αF-helix (C-lobe)		Met231	Ile432

[a] From Refs. [74,81,82].
[b] Part of the Y/HRD sequence.

tissues whereas the β-subunits show a more restricted pattern of expression [85,86].

Uhler et al. [87] reported that mouse Cβ subunits are activated in the nervous system whereas the Cα subunits are the predominant signaling subunit in other tissues. A deficiency of both Cα and Cβ subunits (*Prkaca*$^{-/-}$; *Prkacb*$^{-/-}$) or haploinsufficiency of Cβ (*Prkaca*$^{-/-}$; *Prkacb*$^{+/-}$) is embryonic lethal [59]. In contrast, haploinsufficiency of Cα (*Prkaca*$^{+/-}$; *Prkacb*$^{-/-}$) is associated with the development of spinal neural tube defects in animals that die soon after birth. Moreover, a deficiency of Cα only (*Prkaca*$^{-/-}$) results in growth deficiencies whereas a deficiency of Cβ only (*Prkacb*$^{-/-}$) produces mice that are phenotypically normal. Expression of only one C subunit (α or β) is not compatible with a healthy life. However, expression of any two C subunits (αα, αβ, ββ) is compatible with life.

Amieux et al. [85] reported that the deletion of RIα (*Prkar1a*$^{-/-}$) is embryonical lethal and they demonstrated that the lethality is related to increased basal C subunit activity, which can be partially obviated by deletion of Cα (*Prkar1a*$^{-/-}$; *Prkaca*$^{-/-}$). Deletion of RIβ (*Prkar1b*$^{-/-}$) results in mice that are viable and fertile, but it leads to a deficit in long-term memory whereas deficiency of RIIα (*Prkar2a*$^{-/-}$) leads to mice that are viable and fertile and lack a distinct phenotype [59]. Deletion of RIIβ (*Prkar2b*$^{-/-}$) results in mice that are morphologically normal and fertile, but they have impaired motor coordination and are lean and resistant to diet-induced obesity [59,88,89]. Deletion of both alleles of RIβ, RIIα, or RIIβ, – but not those of RIα – is compatible with life.

The R subunits differ in their subcellular localization, abundance, affinity for the C subunit, sensitivity to cAMP, and specificity for the A-Kinase Anchoring Protein (AKAP) scaffolds [90,91]. AKAPs bind to the regulatory subunits of PKA and position them in appropriate subcellular locations to optimally facilitate signal transduction. Human AKAPs, which make up a family of 15 genes with more than 50 proteins that result from alternative splicing, bind to and restrict PKA in proximity of their substrates where they provide specificity, sensitivity, localization, and timing of physiological responses. Although most AKAPs that have been characterized bind to RII with high affinity, several AKAPs bind RI. Moreover, D-AKAP1 and D-AKAP2 can anchor both RI and RII subunits. Besides PKA, AKAPs interact with protein phosphatases, cyclic nucleotide phosphodiesterases, β-adrenergic and other receptors, PKC, and other proteins to form signaling complexes [90]. That AKAP6 binds to PKA, phosphodiesterase E4D3, Rap1, ERK5, and the cAMP-responsive protein Epac (exchange protein directly activated by cAMP) illustrates the interconnectedness and complexity of cAMP and protein kinase signaling. The existence of AKAPs indicates that protein kinases and phosphatases do not encounter their substrates only by diffusion, but rather as a result of subcellular targeting.

RI isoforms are generally expressed in the cytoplasm of cells whereas RII subunits occur in particulate or membrane-associated cell fractions. The relative ratio of RI and RII subunits also plays a role in cell growth and differentiation. For example, some cancer cells express significantly higher levels of RI than RII [92–94]. In contrast, Beristain et al. [95] reported that the deletion of RIα in mouse mammary epithelium resulted in the development of breast cancers. This deletion is associated with an increased expression of both C subunits and type II regulatory subunits along with the downstream activation of Src. In their analysis of human data sets, these investigators reported that tumors with low RIα and high Src expression were more likely to recur.

Each R subunit contains an N-terminal dimerization/docking (D/D) domain, followed by a flexible linker segment containing a C subunit inhibitory site, and two tandem cyclic nucleotide binding domains (CNB-A and CNB-B) [58]. The inhibitory site of RII contains a phosphorylatable serine and this site functions as both a substrate and an inhibitor. This inhibitory site corresponds to PKA preferred substrates with two arginines, a spacer residue, and a serine that is followed by a hydrophobic residue. The inhibitory RI site contains alanine in place of serine and thus functions exclusively as an inhibitor. Crystal structures of the RIα and RIIβ subunits and holoenzymes have been determined and we consider the structures of the RIIβ family in the next section.

6.2. Structure of the RIIβ regulatory subunit with bound cAMP

Diller et al. [96] solved the structure of the RIIβ monomer containing two molecules of cAMP; the first 129 residues corresponding to the dimerization domain and a large portion of the linker segment connecting the N-terminus to the cyclic nucleotide binding sites, a loop region (326–333), and the C-terminus (413–416) were missing from the electron density. RIIβ contains two interconnected cyclic nucleotide β-barrels, which serve as cores for binding the phosphate of cAMP. Each β-barrel is flanked by α-helices. The αA and αB helices of the CNB-B domain position side chains that interact with cAMP at site A (Fig. 5A). This region serves as a mobile lid for the A site. The lid that seals the B site of RIIβ is provided by the αC-helix of the B-domain, which interacts with the αB-helix of the B domain that in turn interacts with cAMP bound to the A site. This structure allows for any positional or conformational changes from the A-site cAMP to be transmitted through αB to αC and *vice versa*. Sequestration of cAMP in both sites prevents their attack by cyclic nucleotide phosphodiesterases.

The cAMP binding sites possess conserved but distinct features that allow their interaction with their ligands [96]. A conserved peptide segment lies near β-strands 6 and 7 that constitutes a phosphate-binding cassette along with a short phosphate binding αP-helix (Fig. 5A). The cassette begins with a conserved glycine and ends with an invariant alanine; for RIIβ the corresponding sequences are ^{220}GELALMYNTPRAA^{232} (CNB-A) and ^{349}GELALVTNKPRAA^{361} (CNB-B). Conserved arginines (R230 and R359) occur at the bottom of each domain's basket-shaped β-barrel and form salt bridges with the phosphates (Fig. 5C and D). A glycine (220 and 349) and glutamate (221 and 350) form hydrogen bonds with the ribose 2′-hydroxyl group. The N–H group of conserved alanines (223 and 360) form a hydrogen bond with the cAMP phosphates. V173, F189, Y190, I192, and F219 make hydrophobic contacts with cAMP in site A and I320, M323, I339, L351, and Y397 make hydrophobic contacts with cAMP in site B (not shown).

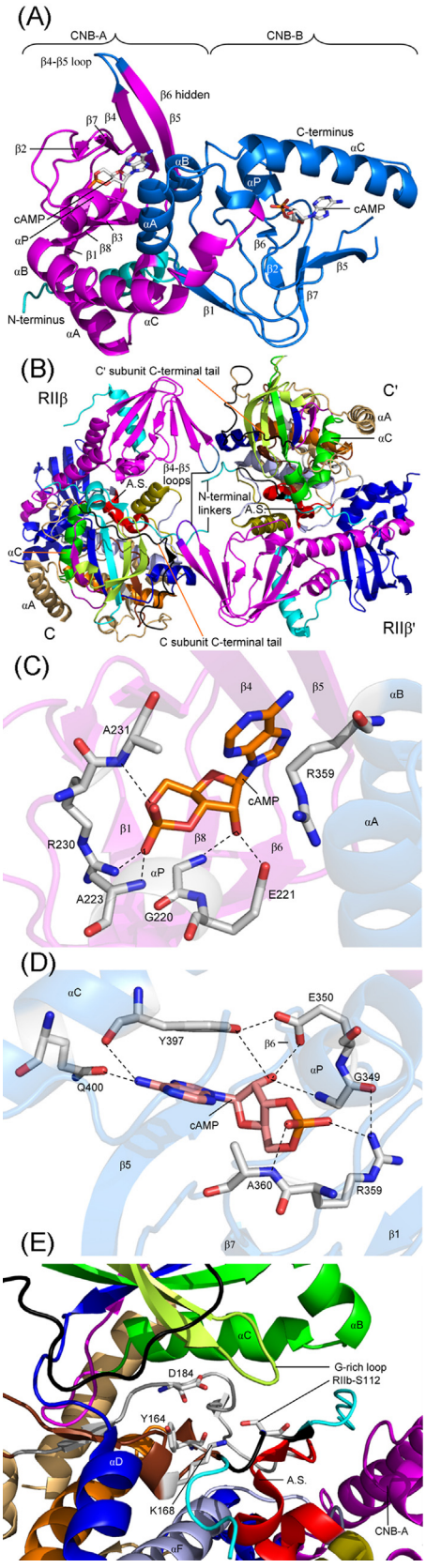

Fig. 5. (A) Two cAMP binding sites in an RIIβ monomer. CNB, cyclic nucleotide binding domain. (B) The protein kinase A $(RII\beta)_2C_2$ holoenzyme made up of a dimer of RIIβ:C and RIIβ′:C′ dimers. A.S., activation segment. (C) cAMP binding site A. (D) cAMP binding site B. (E) Binding of RIIβ to the C subunit. S112 is the phosphorylatable serine of RIIβ. Dashed lines represent polar contacts. A, C, and D were prepared from PDB ID: 1CX4 and B and E were prepared from PDB ID: 3TNP.

In forming the holoenzyme complex, the C subunit binds the R subunit resulting in an R subunit conformational change that leads first to the release of the A-site cAMP (with the concomitant disruption of the A:B domain interface of the R subunits) followed by the cooperative release of cAMP from the B site. This series of distinct conformational states is reversed when the holoenzyme complex dissociates; one cAMP binds to the B site and then another one binds to the A site. Association and dissociation of the type I holoenzyme forms differs in detail from that described here indicating that these mechanisms are isoform specific [58].

6.3. Structure of $(RII\beta)_2C_2$

Zhang et al. [97] determined the tertiary and quaternary structure of a PKA mutant holoenzyme consisting of an $(RII\beta\text{-}R230K)_2C_2$ complex, which was studied owing to the inability of the wild type protein to form satisfactory crystals. Residues 1–103, 122–129, and 394–416 of RIIβ and residues 1–13 of the C subunit were missing in the electron density. The absence of the dimerization domain at the N-terminus of the RIIβ subunits is probably related to the flexible nature of this region. They reported that the R_2C_2 holoenzyme complex makes up a doughnut-shaped solid torus with a dimer of R:C dimers surrounding a central cavity bordered by the N-terminal linkers (Fig. 5B) [97]. Not surprisingly, the holoenzyme structure demonstrates that dramatic changes in the RIIβ subunit occur as it releases cAMP and binds to the C subunit. Two hydrophobic capping residues (Arg381 in the CNB-A site and Tyr397 in CNB-B site, not shown) move away from the cAMP-binding sites in the holoenzyme. Moreover, Arg392 forms a salt bridge with Glu282 and this polar interaction is conserved in all R-subunit isoforms (not shown).

The C subunits in the holoenzyme are separated whereas the R subunit contacts the neighboring R:C heterodimer by using the conserved but isoform-specific β4–β5 loop in the CNB-A domain (Fig. 5B) [97]. This loop is exposed to solvent in the cAMP-bound R subunit. In the holoenzyme tetramer, the β4–β5 loop creates an extensive interface with the opposite R:C heterodimer. The RIIβ β4–β5 loop interacts with the C-terminal tail of the opposite C subunit at residues 320–339 and with the RIIβ N-terminal linker from the opposite heterodimer.

The holoenzyme structure provides a detailed mechanism for the inhibition of the catalytic activity of the C subunit [97]. In each R:C dimer, the RIIβ linker contains an RRASV sequence that corresponds to preferred substrates of PKA. This sequence binds to the substrate-positioning segment of the C subunit near the active site DFG-Asp184 and the catalytic loop Tyr164 and Lys168 (Fig. 5E). In the Type II PKA isoforms, the serine residue (112) undergoes phosphorylation. In the Type I isoforms, the comparable residue is alanine, which does not undergo phosphorylation. As noted previously, the regulation of protein kinase activity varies among the various protein kinases, and the mechanism of PKA regulation by cAMP, its second messenger, is unlike that of any other protein kinase. Owing to the importance of cAMP levels and its downstream regulation that is mediated by many G-protein coupled receptors that either lead to the stimulation or inhibition of adenylyl cyclase, the control of PKA activity is of great physiological importance. Accordingly, PKA participates, *inter alia*, in the regulation of blood pressure, cardiac contractility, memory, neurotransmitter and hormone biosynthesis, glycogen and triglyceride metabolism, and many other physiological processes.

7. Structures of active and inactive Abl protein-tyrosine kinase

7.1. Secondary and tertiary structures of Abl

The small lobe of all protein kinases including Abl is dominated by a five-stranded antiparallel β-sheet (β1–β5) and an important

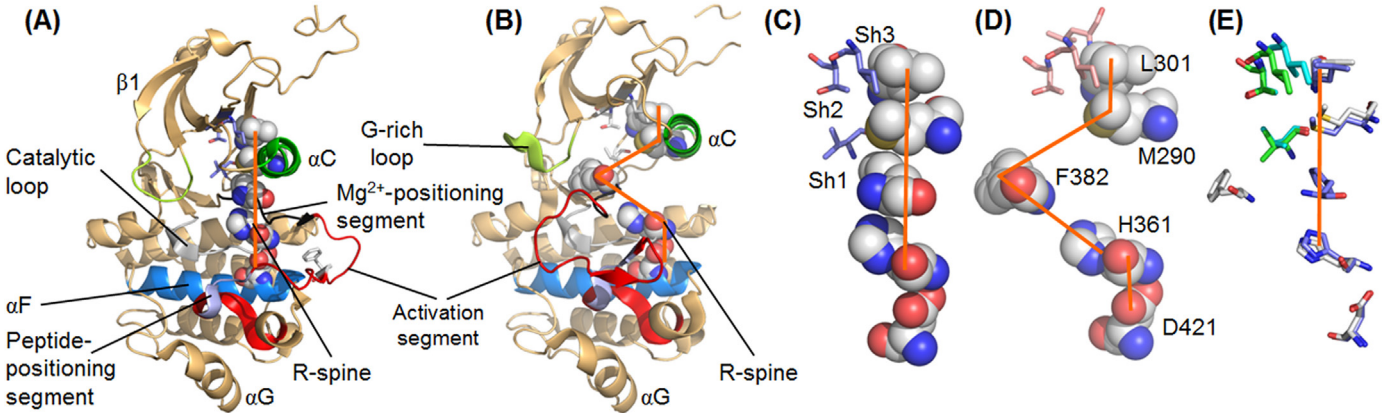

Fig. 6. Structures of the Abl protein kinase domain. (A) Active, (B) Dormant, (C) R-spine active, (D) R-spine dormant. (E) Superposition of active and inactive R-spines. The active and dormant enzyme forms were prepared from PDB ID: 2GQG and 1IEP, respectively.

regulatory αC-helix [74,98]. The first X-ray structure of a protein kinase (PKA) [72,73] contained an αA and an αB-helix proximal to αC (PDB ID:2CPK), but these first two helices are not conserved in the protein kinase family. The large lobe of the Abl protein kinase domain is mainly α-helical with six conserved segments (αD–αI) that occur in all protein kinases [74]. The first X-ray structure of a protein kinase (PKA) possessed a short helix at the end activation segment, which was not named at the time [72,73], but it is conserved in the protein kinase family and is now known as the αEF-helix (Fig. 2A and B). The αF-helix that follows the αEF-helix forms an important hydrophobic core that forms the base of the C- and R-spines.

The large lobe of active Abl kinase contains four short β-strands (β6–β9) [98]. The β6-strand, the primary sequence of which occurs before the catalytic loop, interacts with the activation segment β9-strand. The β7-strand interacts with the β8-strand, the primary structures of which occur between the catalytic loop and the activation segment. The kinase domain of PKA and most active protein kinases contain these nine β-strands. The activation segment of active Abl is open and extends to the right as kinases are viewed classically (Fig. 6A) while that of inactive Abl is compact (Fig. 6B) [98]. The open conformation of the activation segment allows protein substrates to bind to the large lobe while the closed conformation blocks protein substrate binding.

There are two general kinds of conformational changes associated with most protein kinases including Abl. The first conformational change involves the formation of an intact regulatory spine to form an active enzyme [46]. The second conformational change occurs in active kinases such as Abl and PKA as they toggle between open and closed conformations during their catalytic cycles. The open form of the active enzymes bind MgATP and the protein substrate; this is accompanied by the conversion to the closed form as catalysis occurs. After catalysis, phosphorylated protein and then MgADP are released as the enzymes are reconverted to the open form prior to the next catalytic cycle.

Although the tertiary structure of catalytically active protein kinase domains is strikingly similar, Huse and Kuriyan [99] reported that the crystal structures of inactive enzymes reveal a multitude of distinct inactive protein kinase conformations. These investigators indicated that protein kinases assume a dormant conformation in their basal or non-stimulated state and they noted that the acquisition of activity may involve extensive structural changes. Taylor et al. [46] described the process of going from the inactive to active enzyme form (and *vice versa*) as a dynamic switch. As noted previously, the two main regulatory components within the protein kinase domain include the αC-helix of the small lobe and the activation segment of the large lobe.

Less active unphosphorylated Abl does not possess all of the negative regulatory structural elements that occur in many other protein kinases. For example, the αC-helix of Abl is rotated inward, properly positioning Glu286 with Lys271 of the β3-strand (Table 2) as observed in active enzymes [98]. However, dormant Abl assumes the DFG-Asp outward conformation, which corresponds to the inactive state. Moreover, concomitant movement of the DFG-Phe to its inward configuration obstructs the Abl ATP-binding site. Furthermore, the activation segment is in a closed conformation that inhibits protein substrate binding (Fig. 6B). In the active conformation of Abl, the DFG-Asp points inward toward the active site and the activation segment is in the open conformation (Fig. 6A) thus allowing binding of the protein substrate.

7.2. The Abl regulatory spine and shell

An intact regulatory spine is required for the formation of the active protein kinase conformation [74,81]. The R-spine of active Abl resembles that of PKA (Fig. 6A and C) and consists of residues that correspond to those of PKA (Table 3). Because of the conformation with DFG-Asp facing outward and DFG-Phe facing inward, the R-spine in the inactive conformation is broken (Fig. 6B and D). The DFG-phenylalanine (RS2) is far from Met290 (RS3) and H361 (RS1). The superposition of the R-spine and shell residues of Abl show that most of the components of the active and inactive spines are nearly superimposable (Fig. 6E) except for Phe382 (RS2). The residues of the α-carbon atoms of the regulatory spines of active and inactive Abl (excluding Phe382) have a root-mean square deviation of 0.6 Å. In contrast the α-carbon atom of inactive Abl Phe382 is displaced from that of active Abl by 9 Å. Owing to the phosphorylation of the catalytic subunit during its biosynthesis, the regulatory and catalytic spines of PKA occur physiologically in the active state and regulation of enzyme activity is controlled by the regulatory subunits and not by conformational changes within the C subunit.

8. Protein pseudokinases as allosteric regulators

8.1. Pseudokinase properties

In their comprehensive description of the protein kinase complement of the human genome (kinome), Manning et al. [1] reported that 50 of the 518 protein kinases lack amino acid residues important for catalysis. Thus, the absence of the αC-Glu or β3-Lys or alterations in the DFG or HRD signatures was ascribed to catalytically inactive kinases, or pseudokinases. These pseudokinases are scattered throughout the various protein kinase subfamilies, suggesting that they have evolved from diverse active kinases [100].

Moreover, more than two dozen of the pseudokinases have homologues in mouse, worms, flies, and yeast that lack the equivalent catalytic residues. However, numerous experiments demonstrate that many kinases that lack these signature residues possess residual protein kinase activity. For example, calcium/calmodulin-dependent serine protein kinase (CASK), kinase suppressor of Ras (KSR2), avian erythroblastosis oncogene B3 (ErbB3), and Janus kinase 2 (JAK2) possess weak catalytic activity *in vitro* [101].

The occurrence of numerous protein kinases with impaired or non-existent catalytic activity suggests that they possess other essential functions. Reiterer et al. [101] suggested that pseudokinases may modulate the activity of protein kinases by serving as dimerization partners as will be described for ErbB3 below. Alternatively, the impaired kinases may function as integrators by interacting with multiple components of a signaling pathway as described for KSR below. These authors suggested that the pseudokinases may also function as competitors by binding substrates to prevent access to other kinases, phosphatases, or other proteins. They also document studies indicating that impaired kinases may anchor proteins in specific cellular locations. The allosteric interaction of protein kinases with other proteins non-catalytically has added an important feature to the biochemistry and cell biology of the protein kinase superfamily.

8.2. Role of ErbB3 in signaling

Catalytically impaired ErbB3 contains HRNLAARN with an asparagine (N) in place of aspartate (D) in the catalytic loop. The catalytic aspartate (D845) of ErbB1, like that of PKA [78], serves as a base that accepts a proton from the tyrosyl –OH group. Because ErbB3 is kinase impaired, induced homodimer formation may not promote downstream signaling. However, ErbB3 possesses 1/1000th of the autophosphorylation activity of ErbB1 [102] and the possibility exists that the ErbB3 homodimer is catalytically operative. ErbB2 is the favored dimerization partner for all of the other ErbB family members [103,104], and the ErbB2 heterodimer in combination with ErbB1 or ErbB3 exhibit robust signaling activity [105]. The heterodimer consisting of ErbB2, which lacks a ligand, and ErbB3, which is kinase impaired, is surprisingly the most robust signaling complex of the ErbB family. Thus, the ErbB3 protein pseudokinase forms active dimers with other members of the ErbB family to promote efficient downstream signaling and this signaling occurs despite that lack of robust protein kinase catalytic activity.

8.3. KSR and the MAP kinase pathway

Besides the four classical components in the Ras-Raf-MEK-ERK signaling pathway, scaffolding proteins such as Kinase Suppressor of Ras (KSR) play an important role in this signaling pathway. KSR functions as a scaffolding protein that mediates the assembly of the Raf-MEK-ERK module. KSR in *Drosophila* and *C. Elegans* serves as a facilitator of the MAP kinase signaling pathway [106]. Early experiments on KSR function demonstrated that its mutants block Ras signaling so that its designation as suppressor is misleading. Mammals possess one gene for KSR1 and another for KSR2, which are members of the protein serine/threonine kinase family.

In contrast to most active kinases with HRD at the beginning of the catalytic loop, human (UniProtKB Q81VT5/Q6VAB6), *C. Elegans* (UniProtKB Q19380), and *Drosophila* (UniProtKB Q24171) KSR1 and KSR2 contain HKD. Moreover, the essential lysine residue in the β3-strand of most kinases, including the KSR of *Drosophila* and *C. Elegans*, is replaced by an arginine in mammalian KSR1 and KSR2 (human KSR1/2 with R639/R692). Investigators first surmised that these proteins were catalytically inactive owing to the absence of these critical amino acid residues. However, recent work indicates that KSR1 [106] and KSR2 [107] are catalytically active despite these amino acid substitutions. Moreover, KSR1 and 2 allosterically activate components of the Raf-MEK-ERK signaling pathway by an intricate process.

Brennan et al. [107] performed structural and biochemical studies to determine the mechanism of KSR2-stimulated MEK1 phosphorylation as catalyzed by B-Raf. After determining the crystal structure of human MEK1 with rabbit KSR2, they found that the MEK1-KSR2 catalytic sites face each other and the heterodimer interface involves their C-lobe activation segments and αG-helices. Moreover, heterodimer formation occurs with a shift of the KSR2 αC-helix to the active inward conformation. Two KSR2-MEK1 heterodimers form tetramers through a KSR2 homodimer back-to-back interface, which is centered on Arg718. That a KSR2 Arg718His mutation abolishes the activation of MEK1 by B-Raf indicates the importance of the KSR2 dimer interface in the process of MEK1 activation. Brennan et al. hypothesize that B-Raf interacts with the $(MEK1\text{-}KSR2)_2$ heterotetramer to form a MEK1–KSR2–B-Raf ternary complex and then an independent catalytic B-Raf catalyzes the phosphorylation of MEK1 that results in its activation.

Brennan et al. [107] demonstrated that the addition of a kinase-impaired B-Raf Lys483Ser mutant to KSR2-MEK1 increases MEK1 phosphorylation 15-fold as catalyzed by KSR2. The KSR2 inhibitor ASC24 blocks 70% of total MEK1 phosphorylation but less than 10% of MEK1 activation segment Ser118/Ser222 phosphorylation. Sorafenib, a Raf kinase inhibitor, blocks 30% of total MEK1 phosphorylation but over 90% of Ser118/Ser222 phosphorylation as catalyzed by the kinase-impaired B-Raf. They concluded that KSR2 is the major enzyme responsible for increased MEK1 phosphorylation, and this phosphorylation results from the allosteric activation of KSR2 by the B-Raf Lys483Ser mutant.

These experiments indicate that KSR2 possesses catalytic activity despite lacking two canonical signature residues. How is KSR2 activated? B-Raf forms side-to-side dimers that are active [61]. Brennan et al. hypothesize that B-Raf stimulates KSR2 activity allosterically by forming a similar side-to-side heterodimer. Furthermore, these investigators suggest that a KSR2 scaffold promotes the exposure of the MEK1 activation segment for phosphorylation. In summary, these investigators hypothesize that regulatory B-Raf interacts with KSR2 in *cis* to produce a structural change in KSR2 thereby facilitating phosphorylation of MEK1 as mediated by an independent catalytic B-Raf molecule in *trans*.

Hu et al. [106] generated a mouse KSR1 Ala587Phe mutant that cannot bind ATP but stabilizes the C- and R-spines critical for the formation of its active conformation and they used this mutant to assess its scaffold and catalytic functions. Ala587 occurs within the β3-strand and forms part of the catalytic spine. Catalytically active KSR1 binds to C-Raf and produces MEK1 activation. In contrast, the catalytically inactive KSR1 Ala587Phe mutant binds to C-Raf and to MEK1 but does not produce MEK1 activation. Moreover, these investigators found that wild-type KSR1 alone lacks kinase activity. However, co-expression of KSR1 and C-Raf results in KSR1 kinase activity. Their data indicate that MEK1 phosphorylation mediated by C-Raf requires KSR1 catalytic activity. In summary, the experiments of Brennan et al. [106] and Hu et al. [107] provide evidence that both the scaffolding and catalytic activities of KSR are required for MEK1 phosphorylation and activation by Raf.

9. Small molecule inhibitors of protein kinases

9.1. Overview of inhibitors

Because mutations and dysregulation of protein kinases play causal roles in human disease, this family of enzymes has become one of the most important drug targets over the past two decades [108]. Trastuzumab was the first FDA-approved protein kinase

inhibitor (1999); this biologic is a monoclonal antibody that inhibits ErbB2 and is used for the treatment of ErbB2-positive breast, gastric, and gastroesophageal cancers [45,77]. Several other large molecule protein kinase inhibitors have been approved by the FDA. These include panitumumab, cetuximab, and pertuzumab that inhibit EGFR and are approved for the treatment of (i) colorectal cancer, (ii) head and neck cancer, and (iii) metastatic breast cancer, respectively. Ado-trastuzumab emtansine is an antibody–drug conjugate that interacts with ErbB2 and is approved for the treatment of breast cancer [45]. Trastuzumab binds to ErbB2 on tumor cell surfaces; following its internalization, the conjugate emtansine is released and binds to tubulin, thereby disrupting microtubule dynamics and inhibiting cell division and cancer cell proliferation.

Imatinib was the first FDA-approved mechanism-based targeted small molecule protein kinase inhibitor (2001) (www.brimr.org/PKI/PKIs.htm). More than two dozen other orally effective small molecule protein kinase inhibitors have been subsequently approved by the FDA. Cancer is the predominant indication for these drugs, but disease targets are increasing. For example, tofacitinib is a JAK3 inhibitor that is approved for the treatment of rheumatoid arthritis (2012) and nintedanib is a fibroblast growth factor/multikinase inhibitor that is approved for the treatment of pulmonary fibrosis (2014).

Most of the FDA-approved protein kinase inhibitors are competitive with respect to ATP. Targeting the ATP-binding site of protein kinases was not thought to be selective or effective because of the large number of protein kinases and other ATP-requiring enzymes with the likelihood that these binding sites would be indistinguishable leading to numerous side effects [109]. However, the approval of imatinib for the effective treatment of chronic myelogenous leukemia (CML) dispelled this notion. Structural studies reveal subtle differences in the ATP-binding site and contiguous regions that can be exploited to provide specificity for kinase inhibitors. The lesson to be relearned from these observations is that drugs can be tailored to bind to targets that exhibit only subtle differences.

CML is characterized by the activation of Abl kinase following a translocation of its gene to the breakpoint cluster region (BCR) with the attendant formation of the Philadelphia chromosome that encodes a constitutively active BCR-Abl protein kinase chimera [109]. Blockade of a single enzyme is thus efficacious in the treatment of this illness. Most other cancers, however, result from the dysregulation of multiple signaling pathways. The dramatic success in the treatment of CML by an inhibitor of the BCR-Abl kinase is due to a mechanism involving a single biochemical defect. It is important to note that this special characteristic is lacking in nearly all other forms of malignancy and development of inhibitors to single targets has proven to be less effective in treating other cancers as summarized later.

The gatekeeper residue, which is the second shell residue (Sh2) of the R-spine, is an important landmark for characterizing protein kinase inhibitors owing to its ability to influence drug binding. The gatekeeper occurs immediately before the hinge connecting the small and large kinase lobes and it lies next to the 6-amino group of a bound ATP. The ATP-binding site is equivalent to the front pocket of the kinase [48]. Many protein kinase inhibitors bind to their target enzyme by forming 1–3 hydrogen bonds with the hinge residues while interacting with the residues that make up the (i) adenine binding-site and (ii) hydrophobic pockets I or II (Fig. 7A) [110]. When the gatekeeper residue is small (Gly, Ala, Ser, Cys, Thr, or Val), hydrophobic pocket II tends to be large and readily accessible. When the gatekeeper residue is of medium size (Ile, Leu, Met, Gln), hydrophobic pocket II is medium-sized and accessible. When the gatekeeper residue is large (Phe, Tyr), the pocket is small and marginally accessible.

Dar and Shokat [111] classified protein kinase inhibitors as Types I, II, and III. Classical Type I inhibitors bind reversibly to the ATP-binding pocket of protein kinases and exhibit steady-state enzyme competitive inhibition with respect to ATP. Of importance, Type I inhibitors do not require specific spatial arrangements of the αC-helix or the DFG-Asp for their effectiveness and they are able to bind to active and inactive enzyme forms. Type I inhibitors include FDA-approved erlotinib, dasatinib, gefitinib and sunitinib.

Type II inhibitors occupy the adenine site of ATP and the adjacent hydrophobic pocket II (Fig. 7A) [112]. Unlike type I inhibitors, Type II inhibitors bind to the DFG-Asp outward configuration. These inhibitors thus target inactive enzyme conformations. The aspartate or D residue of the DFG conserved sequence is rotated nearly 180° relative to the active state DFG-Asp inward conformation. This rotation then leaves a hydrophobic site (hydrophobic pocket III), which is available for drug binding. The backbone N–H group of the DFG-Asp (Abl Asp381) is available to form a hydrogen bond with a ligand that occupies hydrophobic pocket III. Zuccotto and colleagues [110] introduced the concept of Type I ½ inhibitors, which is a hybrid of the Type I and II classes. Type I ½ inhibitors bind to the hinge residues, the adenine-binding pocket, and hydrophobic pocket II, but with the DFG-Asp inward conformation (not the DFG-Asp outward conformation of Type II inhibitors). Two FDA-approved Type I ½ inhibitors include lapatinib, which is used in the treatment of breast cancer, and vemurafenib, which is used in the treatment of melanoma.

Because Type I ½ and II inhibitors reside partially within the ATP-binding pocket, these inhibitors exhibit steady-state competitive inhibition with respect to ATP. ATP typically forms hydrogen bonds with the first and the third residues of the hinge. The 6-amino group of ATP functions as a hydrogen bond donor with the first hinge backbone residue, and the N-1 nitrogen functions as a hydrogen bond acceptor with the third hinge backbone residue. Type I, I ½, and II inhibitors form variable hydrogen bonds with the hinge residues depending upon the protein kinase and upon the specific inhibitor. The Type I and II inhibitors represent the predominant groups of FDA-approved targeted protein kinase inhibitors.

Type III inhibitors are drugs or compounds that occupy an allosteric site. Historically, allosteric sites refer to other sites, *i.e.*, sites other than the active site [113]. In the case of protein kinases, allosteric sites refer to locations outside of the ATP-binding pocket. Allosteric inhibitors block protein kinase catalytic activity while having no effect on ATP binding. Thus far allosteric inhibitors are not expected to form hydrogen bonds with protein kinase hinge residues. Because ATP is unable to displace allosteric inhibitors, such drugs or compounds are expected to demonstrate steady-state noncompetitive or uncompetitive inhibition. In principle, there are several potential allosteric sites in each protein kinase. Trametinib (GSK1120212, a tetrahydropyridopyrimidine derivative) is a Type III MEK inhibitor that has been approved by the FDA for the treatment of melanoma with *BRAF V600E/K* mutations.

Type IV, or irreversible protein kinase inhibitors, characteristically form covalent bonds with their target enzyme [114]. Owing to safety and toxicity concerns, covalent irreversible drugs are somewhat disfavored as a drug class [115]. However, this concern may be misplaced because aspirin, which exerts its therapeutic effect by covalently modifying Ser530 of cyclooxygenase 1 by acetylation [116,117], has been in the medical therapeutic armamentarium for more than a century. Afatinib, which possesses an α, β-unsaturated carbonyl group, is an irreversible covalent inhibitor of EGFR that is FDA-approved for the treatment of NSCLC [115]. The alkene portion of the drug forms a covalent adduct with Cys797 in the ATP-binding site. It is likely that non-covalent interactions position the small molecule in a productive orientation within the ATP-binding pocket that allows the covalent modification to proceed as the enzyme attacks the electrophilic portion of the drug to form

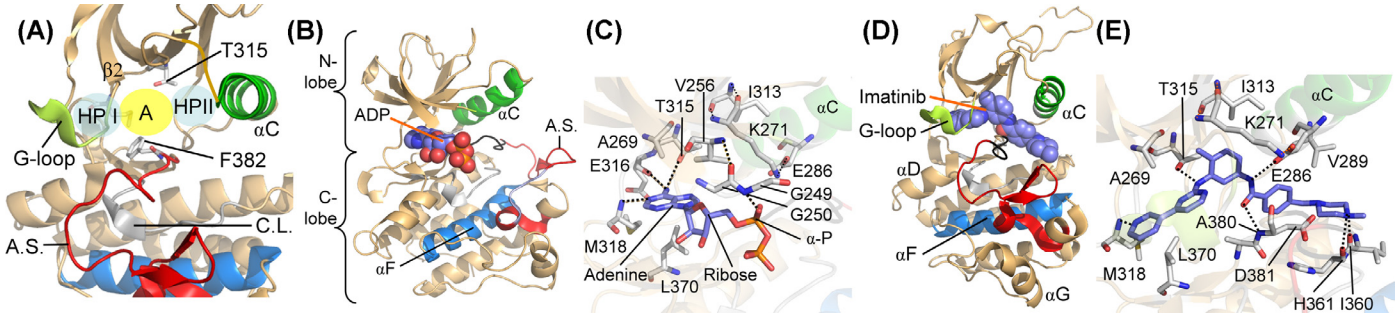

Fig. 7. Binding of ADP and imatinib to Abl. (A) Abl hydrophobic drug-binding pockets I and II (HPI/II). (B) and (C) ADP binding to Abl. (D) and (E) imatinib binding to Abl. A, adenine binding pocket; A.S., activation segment; C.L., catalytic loop. Dashed lines represent polar contacts. (A, D, and E) Prepared from PDB ID: 1IEP. (B and C) Prepared from PDB ID: 2G2I.

a Michael adduct where a Michael reaction represents the addition of a nucleophile (the –SH of cysteine) to an α, β-unsaturated carbonyl compound [118].

9.2. Development of imatinib

Imatinib inhibits BCR-Abl protein-tyrosine kinase and it was the first FDA-approved small molecule protein kinase inhibitor. It was initially used for the treatment of CML (www.brimr.org/PKI/PKIs.htm). Imatinib is known also as STI-571 (Signal Transduction Inhibitor-571), Gleevec (in the United States), and Glivec (in Europe). The chemical precursor of imatinib was discovered by the time-consuming process of testing a large number of compounds for inhibition of PKC *in vitro* [119,120]. Protein kinase C is a serine/threonine kinase that participates in many cellular processes and is implicated in tumor formation. Its activity is regulated by calcium and the diacylglycerol second messengers [121].

The imatinib lead compound contained a phenylaminopyrimidine scaffold that inhibited protein-serine/threonine kinases. The addition of an amide group on the phenyl ring provided inhibitory activity against protein-tyrosine kinases such as BCR-Abl and PDGFR. A subsequent substitution at position 6 of the diaminophenyl ring abolished its PKC inhibitory activity. The additional attachment of a highly polar side chain (*N*-methylpiperazine) increased solubility and bioavailability leading to imatinib, an inhibitor of BCR-Abl, v-Abl, Kit, and PDGFR protein-tyrosine kinases [120].

Initial preclinical laboratory studies targeting BCR-Abl and CML with imatinib were promising. Druker et al. [122] reported that the concentration of imatinib that inhibits BCR-Abl kinase activity 50% *in vitro* is about 25 nM. Moreover, imatinib suppresses the proliferation of BCR-Abl-expressing cells in culture and in animals. They showed that imatinib produces a 92–98% decrease in the number of colonies formed from BCR-Abl cells. Of great importance, imatinib had a minimal effect on colony formation from normal cells. Note that the initial targets of this research were PKC and PDGFR kinase and not the BCR-Abl kinase. Imatinib also inhibits Kit (stem-cell factor/mast-cell factor receptor) protein-tyrosine kinase [123], which increased its therapeutic potential as noted later.

9.3. Comparison of ADP and imatinib binding to Abl

The binding of ADP to Abl resembles that of ATP to PKA. In Abl, the N-1 of adenine makes a hydrogen bond with Met318, which occurs in the hinge that connects the small and large lobes (Fig. 7B and C). The 6-amino group of ADP forms a hydrogen bond with Glu316 of the hinge and also with Thr315, the gatekeeper residue. The α-phosphate of ADP forms a hydrogen bond with Gly249 in the G-rich loop. The adenine base makes hydrophobic contacts with Ile313 and the side chains of Lys271 and Leu370. It also makes hydrophobic contacts with Val256 and Ala269, which are components of the Abl catalytic spine (Table 3).

The binding of inhibitors such as imatinib to Abl have similarities as well as differences when compared with ADP. Nagar et al. [98] and Schindler et al. [124] found that imatinib binds to an inactive conformation of Abl with the DFG-Asp381 in its outward conformation directed away from the active site (Fig. 7E). The phenylaminopyrimidine portion of imatinib is found where the adenine base of ATP normally binds. A nitrogen from imatinib forms a hydrogen bond with the gatekeeper (Thr315) and another nitrogen from the pyridine ring forms a hydrogen bond with Met318 of the hinge. The rest of the compound penetrates into hydrophobic pocket II between the activation loop and the αC-helix, thereby keeping the kinase in an inactive conformation (Fig. 7A and E). Steady-state kinetic studies show that imatinib is a competitive inhibitor with respect to ATP [125], which is consistent with the X-ray studies showing that a portion of the drug is bound to the ATP-binding site.

In the Abl-imatinib binary complex, the DFG-Asp381 of the activation loop points outward and the DFG-Phe382 points inward toward the ATP-binding site (not shown). Asp381 binds Mg^{2+} (1) of the MgATP substrate in the active enzyme, but this salt bridge cannot occur in Abl-imatinib owing to the rotation of the activation segment. Moreover, the spatial arrangement with Phe382 pointing toward the ATP-binding site is required for imatinib binding (not shown). The activation segment adopts a conformation in which the region surrounding Tyr393 (an activating phosphorylation site) mimics substrate binding to the enzyme and thereby blocks the binding of exogenous proteins. In the dormant non-phosphorylated enzyme, Tyr393, which is folded into the active site of Abl, forms a hydrogen bond with Asp363 (the HRD catalytic base) [98,124].

A total of 21 amino acid residues of the Abl catalytic domain interact with imatinib forming six hydrogen bonds (Fig. 7E). Additional interactions involve van der Waals contacts between the protein and the aromatic rings of the inhibitor [98]. The complementary fit limits any modification of the inhibitor without compromising its binding affinity. Once imatinib is bound to Abl, it prevents the activation loop from changing conformation. As a Type II inhibitor, imatinib is effective only when Abl is unphosphorylated and in its dormant DFG-Asp outward/DFG-Phe inward conformation.

In CML, BCR is fused with Abl thereby eliminating the inhibitory interaction of the physiological Abl N-terminus with the large lobe, thus resulting in constitutively active Abl [126]. Accordingly, the activation loop in BCR-Abl would be in the open conformation and phosphorylated. Given that imatinib cannot recognize this open conformation, how does imatinib achieve its great inhibitory effect? Huse and Kuriyan hypothesized that the phosphorylation state is dynamic and cellular phosphatases catalyze the removal of the BCR-Abl activation segment phosphate [98]. Only when the

activation loop is transiently dephosphorylated can imatinib bind and block Abl protein kinase activity. Most experiments evaluate net phosphorylation site stoichiometries, and this proposal by Huse and Kuriyan [99] suggests that additional experiments addressing phosphate turnover may contribute to our understanding of the dynamics of phosphorylation–dephosphorylation under physiological conditions.

9.4. The unusual clinical efficacy of imatinib

Imatinib is a multikinase inhibitor that blocks the activity of BCR-Abl, Kit, mutant Kit, and PDGFR [109]. Imatinib is approved for the treatment of Ph^+ (i) CML and (ii) ALL. Imatinib is also approved for the treatment of *KIT*-mutant positive GIST and myelodysplastic diseases with *PDGFR* gene rearrangements and hypereosinophilic syndrome with FIP1L1-PDGFRα fusion proteins (www.brimr.org/PKI/PKIs.htm).

Small molecule protein kinase inhibitors are generally well tolerated. For example, the side effects of imatinib used in the treatment of CML are usually mild and rarely lead to discontinuance of therapy [127]. In contrast, anticancer drugs such as methotrexate, which are administered intravenously at a maximum tolerated dose, are cytotoxic and wreak havoc on the recipient [128]. The term pharmaceutical is derived from *pharmakon*, which is Greek for poison. Side effects of many cytotoxic drugs such as methotrexate include nausea, vomiting, myelosuppression, mucositis, and hepatotoxicity.

Targeted small molecule protein kinase inhibitors, however, are not panaceas. Severe side effects may occur in 1–2% of patients [45]. The cost of these protein kinase inhibitors approaches or exceeds $10,000 per month despite their inability to greatly prolong life [129]. Moreover, resistance to nearly all of the FDA-approved protein kinase inhibitors occurs within several months to a few years. The sole exception thus far appears to be the treatment of CML with imatinib. Hughes et al. [130] reported that the overall seven-year survival rate was 86% in those people who received the drug. Additionally, Khorashad and Deininger [131] estimated that 60–80% of people experience a clinical benefit that lasts more than a decade. Such long-term benefits in the treatment of CML with imatinib are truly unique among small molecule protein kinase inhibitors.

10. Resistance to imatinib and other targeted protein kinase inhibitors

10.1. Imatinib resistance

10.1.1. Ph^+ chronic myelogenous leukemia

Rowley discovered that the Philadelphia chromosome is formed from a reciprocal translocation t(9;22)(q34;q11.2) that results in a lengthened chromosome 9 and a shortened chromosome 22 (the Philadelphia chromosome) [132]. The Philadelphia chromosome occurs in about 95% of chronic myelogenous leukemia patients. The BCR-Abl oncogene results from this translocation where Abl is the human ortholog of the murine Abelson leukemia virus, which was originally on chromosome 9, and BCR refers to the breakpoint cluster region, which was originally on chromosome 22 [109,133]. As a result of the formation of the fusion protein encoded by the Philadelphia chromosome, the physiological N-terminal inhibitory domain of Abl is eliminated thereby leading to an activated BCR-Abl kinase, which stimulates multiple signaling pathways and is the dominant factor in the pathogenesis of the disease.

The natural history of CML is one of steady deterioration with a median survival of three years without treatment. The disease consists of a chronic phase, an accelerated phase, and a blastic phase [131]. After a chronic phase of a few years duration, about half of CML patients enter an accelerated phase lasting 6–12 months, which terminates in a blast crisis. The other half of CML patients enter the blast crisis directly and under both conditions the crisis behaves like an acute leukemia, which rapidly progresses to death. Disease progression is most likely due to epigenetic changes and new mutations that occur after the BCR-Abl translocation.

As noted by Winer et al. [134] "Biologically, the cancer cell is notoriously wily; each time we throw an obstacle in its path, it finds an alternate route that must then be blocked". Imatinib has considerably better therapeutic properties than most approved small molecule protein kinase inhibitors in terms of (i) its effectiveness against CML (greater than 95% complete hematological response [135]) and (ii) the relative lack of resistance when compared with other drugs and neoplastic diseases. However, several mechanisms of resistance to imatinib have been described [136]. These can be classified as BCR-Abl-dependent or independent. The first group includes amplification or overexpression of BCR-Abl or point mutations in the Abl protein kinase domain [137] The second group includes decreased drug uptake, increased drug efflux, or upregulation of signaling modules such as the Ras-Raf-MEK-ERK and Src family kinase pathways [138]. BCR-Abl-dependent processes, particularly point mutations, are the most common mechanisms of imatinib resistance in CML. More than 100 different mutations have been described, and they occur in several enzyme locations including the ATP-binding site, the catalytic loop, the activation segment, and amino acids that make direct contact with imatinib.

The following four amino acid replacements account for about 60% of the mutations in BCR-Abl found at the time of CML relapse: Thr315Ile, Tyr253Phe, Glu255Lys/Val, and Met351Thr [137]. The side chain alcohol of the Thr315 gatekeeper hydrogen bonds with imatinib, but this interaction cannot occur with the isoleucine mutant. Construction of Abl containing this alteration showed that the enzyme is insensitive to imatinib. Tyr253 makes van der Waals contact with imatinib; substitution of phenylalanine at this position leads to a resistant form of the kinase. It is unclear how this mutation leads to resistance because phenylalanine is expected to form a similar van der Waals contact. Glu255 is located within the ATP-binding site of the kinase; mutations that convert glutamate to lysine or valine lead to enzymes that are insensitive to imatinib. Met351 occurs in the αE-helix of the C-lobe, but it is unclear how substitution of a threonine at this location imparts resistance to imatinib.

Owing to the development of imatinib-resistant BCR-Abl mutants, second generation inhibitors including dasatinib, nilotinib, and bosutinib have been developed and approved by the FDA for the treatment of BCR-Abl-positive CML (www.brimr.org/PKA/PKIs.htm) [138]. Ohanian et al. [138] suggested that either dasatinib or nilotinib should be given as first-line therapy because each of them is more potent and has a higher optimal response rate when compared with imatinib. Subsequent studies indicate that dasatinib or nilotinib are marginally more effective than imatinib [139]. Because imatinib is scheduled to come off of its patent in 2015, the expected lower cost of a generic equivalent along with its general overall effectiveness favor its continued therapeutic use in the primary setting. Unfortunately, none of these three second-generation drugs is effective against the BCR-Abl Thr315Ile gatekeeper mutation. However, ponatinib is a third generation BCR-Abl inhibitor that is approved by the FDA for the treatment of CML patients with the gatekeeper mutation. In summary, imatinib is a well established and effective drug for the long-term treatment of Ph^+ CML.

10.1.2. Ph^+ acute lymphoblastic leukemia

The BCR-Abl fusion protein that occurs as a result of the formation of the Philadelphia chromosome occurs in 3–4% of pediatric and about 25% of adult ALL cases [140]. The pathogenesis of ALL is more intricate than that of CML owing to the occurrence of mutations in addition to the BCR-Abl translocation. For example,

mutations or deletions of the *IKZF1* gene occur in 70–80% of Ph^+ ALLs. The gene product is a zinc-finger protein that is an important regulator of normal lymphocyte development [141]. Other commonly occurring mutations include those of *PAX5*, *EBF1*, or *CDKN2A/B*. Most likely as a result of the concurrent mutations and in distinct contrast to CML, monotherapy of ALL with imatinib is generally ineffective with complete response rates of only 20% with subsequent relapses occurring within 58 days [141].

Historical ALL treatments include the use of cytotoxic drugs (*e.g.*, cytarabine, doxorubicin, methotrexate, vincristine, and other agents) and stem cell transplants [141]. In contrast to the use of either cytotoxic agents or imatinib, the combination of these two therapies induces complete remission in greater than 95% of patients with overall three-year survival rates of 50% [140,141]. However, the combined treatment of ALL with imatinib and cytotoxic agents is less effective than the treatment of CML with imatinib monotherapy.

The mechanisms of imatinib resistance in ALL is similar to that of CML with BCR-Abl-dependent and independent mechanisms [141]. The dependent mechanisms involve the same mutations of BCR-Abl that occur in CML including Glu255Lys, G-loop mutations, and the Thr315Ile gatekeeper mutation. BCR-Abl-independent mechanisms include decreased cellular drug uptake and upregulation of alternative signaling pathways including the Src family kinases Lyn, Hck, and Fgr. Accordingly, Src family kinase inhibitors including bosutinib or vandetanib (www.brimr.org/PKI/PKIs.htm) represent possible alternative or concurrent therapies that address this mechanism of resistance.

Dasatinib is a second-generation BCR-Abl inhibitor that is effective against many of the ALL imatinib-resistant mutants with the exception of that of the gatekeeper (Thr315Ile). Ponatinib is a third generation BCR-Abl inhibitor that is effective against most of the imatinib-resistant mutants including the gatekeeper mutation (www.brimr.org/PKI/PKIs.htm). Current clinical trials are underway to determine the most effective treatments for Ph^+ ALL including protocols combining protein kinase inhibitors and cytotoxic agents along with the use of therapeutic monoclonal antibodies [141,142]. In summary, resistance to imatinib monotherapy occurs within 60 days and resistance to combination imatinib and cytotoxic therapy in the treatment of Ph^+ ALL usually occurs within three years, which is in contrast to the more enduring response of Ph^+ CML with imatinib alone.

10.1.3. KIT *mutations in gastrointestinal stromal tumors*

Gastrointestinal stromal tumors (GIST) are sarcomas that arise from the interstitial cells of Cajal, which play a role in intestinal motility. The clinical course of GIST is extremely variable ranging from benign to malignant [143]. The standard treatment for localized GIST is surgical removal. Recurrence after surgical resection occurs in up to 90% of people with tumors greater than 10 cm in diameter. In people with recurrent disease, the results of surgery, radiation therapy, and cytotoxic chemotherapy are poor. However, the use of targeted Kit therapy with imatinib greatly improves the outcome, but resistance is common.

In a pioneering study, Hirota et al. [144] found that Kit mutations are prevalent in GIST. About 80% of GIST patients harbor an activating mutation in *KIT* and 5–10% possess activating mutations in PDGFRα (*PDGFRA*) [143,145]. The remainder lack either of these mutations. *KIT* mutations in exon 11, which corresponds to the juxtamembrane domain (between the plasma membrane and the protein kinase domain), occur in about 67% of all cases of GIST [145,146]. These findings indicate that the juxtamembrane segment is normally inhibitory. Although some of these *KIT* mutations involve amino acid substitutions, most of these consist of deletions of two to sixteen residues. About 17% of the *KIT* mutations occur in the extracellular domain (exon 9) and about 2% involve a Lys642Glu substitution in the αC-loop of the small lobe (exon 13). Another 2% of the *KIT* mutations involve residues in the activation loop of the large lobe (exon 17). In contrast to Kit, the majority of *PDGFRA* mutations occur within the activation loop and a minority involve the juxtamembrane segment.

Imatinib is effective in the treatment of a significant proportion of people with GIST [145,146]. About 90% of those individuals with *KIT* mutations respond favorably to imatinib, but 10% exhibit primary resistance and fail to respond [147]. Of the 10% of patients with primary resistance, about half of them possess *PDGFRA* mutations, one-third have both wild type *KIT* and *PDGFRA*, and the mechanisms of resistance in the remaining 1/6th are unknown. Although imatinib is also an inhibitor of PDGFRα, gain-of-function mutations of this receptor involve the activation loop and most of these mutants are resistant to imatinib.

Patients with secondary resistance are those who first respond to imatinib and then become refractory with a median overall survival of about five-years [148]. As in the case of mutations of BCR-Abl in CML that lead to drug resistance, similar resistance mutations of *KIT* occur in about 60% of those undergoing imatinib treatment of GIST [149]. Mutations in Kit that confer secondary imatinib resistance include the Thr670Ile gatekeeper mutation in exon 14. The Thr670 hydroxyl group forms a hydrogen bond with imatinib [150], but Ile670 cannot form such a bond. As noted previously, a similar Thr330Ile mutation occurs in BCR-Abl in CML that confers drug resistance [109]. Other *KIT* drug-resistant mutants include V654A (exon 11) in the αC-β4 loop and D816/A/G/H/V, D820/A/W/G/Y, N822H/K, and Y823D (exon17) and A829P (exon 18) in the activation segment [148].

Sunitinib, which is an inhibitor of Kit, PDGFR, VEGFR1/2/3, Flt3 and RET, has been approved as a second-line therapy for imatinib-resistant GIST patients (www.brimr.org/PKI/PKIs.htm). Regorafenib is a KIT/multikinase inhibitor that is in clinical trials for the treatment of GIST; regorafenib is FDA-approved for the treatment of renal cell, differentiated thyroid, and hepatocellular carcinomas. Additional GIST clinical trials are underway to determine optimal treatment protocols involving surgery, imatinib, and other targeted protein kinase inhibitors. In addition to second-site *KIT* resistance mutations, Kit gene amplification and activation of bypass pathways are responsible for secondary imatinib resistance [151]. As with all targeted protein kinase inhibitors, the mechanisms of secondary resistance for a significant proportion of GIST patients is unknown. In summary, the treatment of metastatic and recurrent GIST with imatinib greatly improves the outcome compared with cytotoxic therapies, but resistance occurs in half of the patients within five years. Imatinib therapy for GIST is more effective than that for ALL, but not as effective as that for CML.

10.2. Crizotinib resistance

The EML4-ALK fusion protein plays a fundamental role in the development in about 5% of NSCLCs [152] and crizotinib has been approved by the FDA for the treatment of these malignancies. The overall response rate for crizotinib in the treatment of ALK-positive NSCLC patients was 57% ($n = 82$), which is much lower than the response rate for imatinib of greater than 95% in CML. Katayama et al. [153] reported that crizotinib resistance occurred in all ALK-positive NSCLC patients ($n = 18$) studied with a range of 4–34 months and a median of 10.5 months. Like drug-resistant mutations in BCR-Abl, nearly a dozen different mutations in ALK fusion proteins have been discovered that confer crizotinib resistance. However, only 28% of crizotinib resistance in patients was due to mutations of the oncoprotein or to *EML4-ALK* gene amplification. The other possible modes of resistance are related to upregulation of bypass signaling pathways or to unknown mechanisms. Ceritinib is a second generation drug that is approved for the

treatment of ALK mutants (www.brimr.org/PKI/PKIs.htm) and several other drugs are in clinical trials, but additional strategies will have to be employed to address upregulation of bypass pathways.

10.3. Erlotinib and gefitinib resistance

Dysregulation of EGFR protein kinase activity has been implicated in the oncogenic transformation of various types of cells [154]. For example, amplification or activation of EGFR has been observed in NSCLC. A Leu858Arg or a Gly719Ser mutation or small in-frame deletions of exon 19 corresponding to the αC-helix lead to the expression of activated EGFRs in NSCLC that are sensitive to gefitinib and erlotinib [155]. Although the FDA initially approved gefitinib for the treatment of NSCLC with *EGFR* mutations [156] for only two years (2003–2005), this approval was reinstated in 2015. Moreover, gefitinib is officially approved for this treatment in dozens of countries worldwide. Erlotinib is approved by the FDA for the treatment of NSCLC bearing *EGFR* mutations [157]. Nevertheless, people initially responding to erlotinib therapy invariably develop resistance, thereby limiting median progression-free survival to 14 months and a median overall survival of 27 months [158]. Afatinib is an irreversible EGFR inhibitor that was approved for the treatment of NSCLC in the United States, Europe, Taiwan, Japan and other countries in 2013, but this comes with the challenge that the tumors will develop resistance [159,160].

Unlike BCR-Abl and ALK with numerous resistance mutations, secondary mutations of EGFR oncoproteins that confer drug resistance thus far involve only the gatekeeper residue (Thr790). What is the mechanism for this resistance? One might expect that the replacement of the threonine gatekeeper of EGFR with the larger methionine might block the access of hydrophobic pocket II adjacent to the adenine-binding site (Fig. 7A). Surprisingly, this does not occur. Thus, Yun et al. [155] reported that the affinity of gefitinib is greater for the *EGFR* Thr790Met gatekeeper mutant (K_i = 4.6 nM), the activated Leu858Arg mutant (K_i = 2.4 nM), and the drug-resistant Leu858Arg/Thr790Met double mutant (K_i = 10.9 nM) than for the wild type enzyme (K_i = 35.3 nM). Moreover, the Leu858Arg mutant has a K_m for ATP of 148 μM, which is increased when compared with the wild type (K_m = 5.2 μM), the Thr790Met gatekeeper mutant (K_m = 5.9 μM), and the Leu858Arg/Thr790Met drug-resistant double mutant (K_m = 8.4 μM). Based on these K_m data, the *EGFR* Thr790Met gatekeeper mutation decreases gefitinib sensitivity of the Leu858Arg mutant by increasing the ability of ATP to more effectively compete with drug binding during catalysis. Presumably these studies apply to the mechanism of erlotinib binding to EGFR and to erlotinib resistance.

Mutation of the gatekeeper of EGFR from threonine to methionine contributes to oncogenesis and drug resistance by another process; such a mutation increases EGFR protein kinase catalytic activity. Azam et al. [161] reported that the substitution of isoleucine or methionine for the threonine gatekeeper in Abl, EGFR, PDGFRα/β, and Src protein kinases enhances enzyme activity. They reported that the methionine mutation increases activity more than the isoleucine mutation. As noted previously, the gatekeeper residue occurs near the top of the hydrophobic R-spine, and these investigators ascribed enzyme activation to the ability of the hydrophobic gatekeeper to strengthen the R-spine and promote formation of the active conformation of the protein. This proposal agrees with a subsequent report indicating that the gatekeeper (Sh2) stabilizes the R-spine and thereby supports the catalytic activity of PKA [74].

10.4. Vemurafenib resistance

Vemurafenib is an orally effective drug that targets the B-Raf Val600Glu mutant in melanoma with an overall response rate of about 69% (n = 16) [162]. Tuma reported that secondary or developed drug resistance occurs between 2 and 18 months with a median duration of response of 6.2 months [163]. This resistance occurs despite the absence of secondary mutations in B-Raf [48] and this is an unusual property among targeted protein kinases. The mechanisms of B-Raf inhibitor resistance include (i) *NRAS* mutations, (ii) overexpression of COT (MAP3K8 of the STE group), (iii) increased PDGFRβ activity, (iv) increased IGF-1R activity, (v) increased expression of the B-Raf Val600Glu mutant (the primary drug target), and (vi) the emergence of an alternatively spliced B-Raf Val600Glu enzyme that is resistant to vemurafenib [48]. This brief survey indicates that the biology and behavior of each type of tumor is unique with distinct pathways leading to drug resistance.

11. Development of protein kinase inhibitors

11.1. The role of serendipity in drug development

Imatinib was developed as a PDGFR inhibitor, but its initially approved therapeutic targets included BCR-Abl and Kit [109,164]. Imatinib was approved in 2001 for the treatment of BCR-Abl-positive CML and in 2002 for the treatment of GIST with *KIT* mutations. The drug has subsequently been FDA-approved for the treatment of a variety of other diseases including adults with (i) relapsed or refractory Ph^+ ALL, (ii) myelodysplastic/myeloproliferative diseases associated with *PDGFR* gene rearrangements, (iii) aggressive systemic mastocytosis, (iv) hypereosinophilic syndrome and/or chronic eosinophilic leukemia, and (v) unresectable, recurrent and/or metastatic dermatofibrosarcoma protuberans (www.accessdata.fda.gov/scripts/cder/drugsatfda/).

Crizotinib was initially developed as a c-Met inhibitor, but it was later found to inhibit ALK [165]. Sorafenib is approved for the treatment of NSCLC bearing the EML-ALK fusion protein. Sorafenib was initially developed as a Raf inhibitor, but it inhibits several protein kinases including Flt-3, Kit, Raf, Ret, PDGFR, and VEGFR (www.brimr.org/PKI/PKIs.htm) [166]. This drug is approved for the treatment of renal cell carcinoma and for hepatocellular carcinoma. Its effectiveness against renal cell carcinoma may involve its inhibition of VEGFR and angiogenesis and its effectiveness against hepatocellular carcinoma may involve the combined inhibition of VEGFR and of Raf in the Ras-Raf-MEK-ERK signal transduction pathway.

In contrast to these examples where the therapeutic drug target differs from that of the original drug discovery program, other drug development programs were successful in fabricating compounds against an initial target. For example, vemurafenib was developed as an inhibitor of B-Raf [167] and is approved for the treatment of melanomas bearing the oncogenic Val600Glu mutation. Additionally, erlotinib and gefitinib were developed as inhibitors of the EGFR family [168,169] and are approved in dozens of countries for the treatment of NSCLC bearing specific oncogenic *EGFR* mutations.

11.2. Timelines of drug development

Gerber and Mina [170] compared the timelines of the development of crizotinib, erlotinib, imatinib, and vemurafenib and contrasted the interval between drug target discovery and the documentation of drug target inhibition. After the discovery of BCR-Abl protein kinase in 1984, imatinib was shown to block this activity 16 years later (2000) leading to its FDA approval in 2001 [109,119]. That EGFR participates in malignant transformation was known by 1995 [171] and its inhibition by gefitinib (2003) and erlotinib (2004) was established along with their FDA approvals [172]. The role of *BRAF* mutations in melanoma was discovered in 2002 [173] and clinically effective inhibitors were identified six years later (2008) [167]. Subsequently vemurafenib was approved for the treatment of melanoma in 2011 [174]. That Kit participates

in the pathogenesis of GIST was discovered in 1998 [144] and a clinically effective candidate (imatinib) was identified and approved within four years (2002) [145]. As noted above, imatinib was initially approved for the treatment of CML [109,119].

Soda et al. [175] reported the discovery of EML4-ALK fusion proteins in a small proportion of NSCLC in 2007. At the same time, Christensen et al. [176] and Zou et al. [177] were examining the effects of crizotinib on tumor growth mediated by activated c-Met and ALK in cells and in animals. Crizotinib was shown to be clinically effective against EML4-ALK positive NSCLC in 2010 [178,179] and the FDA approved it for first-line therapy in August 2011. It is unlikely that much can be done to increase the rate of translation from the laboratory to the clinic that occurred with crizotinib (2007–2011) because of the time required for clinical trails to demonstrate therapeutic efficacy, but the experience gained from various protein kinase drug discovery programs undoubtedly aids in this process.

12. Epilog

Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) result in a disease with variable signs and symptoms [180]. HIV is a retrovirus that primarily infects components of the human immune system such as $CD4^+$ T cells, macrophages, and dendritic cells. It directly and indirectly destroys $CD4^+$ T cells. HIV1 and HIV2 contain genes that encode the proteins required for virus replication: *gag* encodes the proteins that form the core of the virion, *pol* encodes reverse transcriptase, integrase, and the protease that is responsible for protein processing while *env* encodes the envelope glycoproteins [180].

Treatment of AIDS with single reverse transcriptase inhibitors rapidly leads to resistance owing to viral mutations [181]. Current treatments with combinations of drugs have led to a dramatic decrease in the incidence of drug resistance. A combination of three reverse transcriptase inhibitors such as efavirenz/tenofovir/emtricitabine, or a combination of two protease inhibitors and two reverse transcriptase inhibitors such as ritonavir/atazanavir/tenofovir/emtricitabine, or a combination of an integrase inhibitor plus two reverse transcriptase inhibitors such as raltegravir/tenofovir/emtricitabine are clinically effective recommended standards of care (aidsinfo.nih.gov). Although the precise mechanisms that enable combinations of drugs to escape resistance are unclear, Das and Arnold suggested that mutations that produce resistance to one reverse transcriptase inhibitor may enhance sensitivity to another inhibitor; this hypothesis provides a possible molecular basis for the long-term clinical utility of drug combinations in the treatment of AIDS [182].

Owing to the near universal and expected development of targeted protein kinase inhibitor resistance in the treatment of neoplastic disorders, new approaches are required to overcome this therapeutic challenge. One possibility is to combine a targeted protein kinase inhibitor with traditional cytotoxic therapy; this strategy was effective in the treatment of Ph^+ ALL as noted above [140,141]. Another strategy is to dispense multiple drugs that inhibit one or more protein kinases [183]. To determine whether the co-administration of two or three drugs that are directed at the same enzyme will prevent drug resistance in neoplastic cells will require clinical studies. As noted above, this strategy has been successful in the treatment of AIDS. However, a compounding factor is that AIDS is caused by a single entity and is susceptible to pharmacological inhibition whereas the pathogenesis of cancers is multifactorial and may be less amenable to this approach.

To combat the resistance to a single protein kinase inhibitor, the use of multiple inhibitors at the start of therapy may be more effective than using monotherapy while resistance develops. The selection of which drugs to co-administer that target the same protein kinase is problematic. One possibility is to use a combination of Type I and Type II inhibitors that target different forms of a protein kinase. For example, dasatinib and imatinib are approved for the treatment of CML (www.brimr.org/PKI/PKIs.htm). The former is a Type I inhibitor and the latter is a type II inhibitor that binds to the DFG-Asp outward BCR-Abl conformation [98,124]. Clinical trials are underway comparing the two drugs in the treatment of newly diagnosed CML (NCT00070499 and NCT00481247), but none are underway using both drugs together. Both sunitinib and sorafenib are approved for the treatment of renal cell carcinoma. The former is a Type I inhibitor and the latter is a Type II inhibitor and both of these drugs are multikinase inhibitors that target the VEGFR family of protein kinases. A clinical trial is underway evaluating the sequential use of the two drugs (NCT01481870), but none are underway using both drugs together.

Another tactic is to use a combination of drugs that inhibit the same signal transduction pathway. For example, Johnson et al. [184] reported that the combination of dabrafenib (a B-Raf inhibitor) and trametinib (a MEK1/2 antagonist) was somewhat more effective in treating people with metastatic melanoma than dabrafenib monotherapy. As noted above, another therapeutic possibility is to co-administer combinations of drugs that inhibit the main oncogenic driver, *e.g.*, inhibition of ErbB2 with both trastuzumab and pertuzumab in breast cancer [185]. These biologics have different mechanisms of action. Trastuzumab disrupts growth-factor-independent ErbB2/ErbB3 dimer formation while pertuzumab inhibits growth-factor-dependent association of ErbB2 with other ErbB family members. This combination has proven successful in the treatment of ErbB2-positive breast cancer along with docetaxel, a cytotoxic agent.

The analysis of tumor biopsies from patients who have developed resistance to a variety of drugs has been used to determine resistance mechanisms and the results can be used as a guide for treatment prior to the development of resistance in newly diagnosed patients. One common type of mechanism is the activation of bypass pathways [183]. While the initial target is inhibited, such as EGFR, bypass receptor protein-tyrosine kinases such as Axl, ErbB2, FGFR, IGF-1R, or c-Met may be up-regulated. The bypass receptors activate the Raf-MEK-ERK and PKB/Akt pathways that are also downstream from EGFR. Thus, targeting the primary oncogenic driver along with a bypass receptor represents an attractive possibility. Owing to differences in resistance of the same type of tumor, it is difficult to choose among these alternative targets. Niederst and Engelman [183] suggested that alternating and intermittent dosing of different protein-tyrosine kinase receptor inhibitor combinations be given in a proactive manner (before resistance has developed).

Cancers are complex entities with a variable genetic landscape [186]. Moreover, cancers are heterogeneous and the component tumor cells can exhibit variable properties [187]. This tumor heterogeneity occurs as a result of branched development and clonal evolution during cancer progression. One consequence of this heterogeneity is that the therapeutic response within a given tumor can be variable, a property that may contribute to resistance development. While many cells may undergo apoptosis as a result of targeted drug treatment, others cells may already have or are prone to develop resistance. Accordingly, the tumor may shrink initially during therapy, but resistant clones within the tumor may expand and give a clinical picture of relapse. The understanding of tumor progression and heterogeneity prompts changes in clinical practice and research approaches to identify biomarkers or other characteristics that can be used to provide more durable therapeutic responses.

Multiple clinical trials are examining the effectiveness of combinations of drugs that inhibit EGFR and c-Met, IGF-1R, or FGFR in

breast cancer (www.ClinicalTrials.gov). Clinical trials using EGFR inhibitors along with ErbB3 antibodies are also underway. The choice of which drugs and whether they should be given together or sequentially along with the identification and use of appropriate biomarkers during the course of treatment will require considerable developmental work. Some studies indicate that small subpopulations of cells in drug-naive (previously untreated) tumor samples harbor amplifications or mutations that will eventually lead to drug resistance [183,187]. Such identification prior to the development of frank resistance would help take some of the guesswork out of formulating effective drug combinations.

One drawback with the strategy of using combinations of targeted drugs is the added expenditure of such regimens. With a single targeted drug costing $10,000 per month in the United States, the fee for combinations of drugs promises to be two or more times this amount and this charge may be prohibitive. The high fee for both orally effective small molecule inhibitors and monoclonal antibodies that are directed against oncogenic pathways contributes to the financial toxicity of cancer drugs [188]. As a consequence of financial distress, patients are noncompliant and may skip appointments. Furthermore, they may take less than the prescribed amount of a medication and a significant portion in one study (24%) avoided filling prescriptions altogether [188]. Moreover, health insurance does not eliminate financial distress or health disparities among cancer patients because of the considerable expense of co-payments. Unfortunately for the patient and society, the most common cause of personal bankruptcy in the United States is medical debt owing to the high cost of drugs [129].

There are literally hundreds of protein kinase inhibitors in various stages of clinical development worldwide. As a result of numerous preclinical and clinical drug development programs, more than two dozen orally effective mechanism-based small molecule protein-kinase inhibitors were approved by the FDA since 2001 (www.brimr.org/PKI/PKIs.htm). Although it may be counterintuitive, the majority of these drugs lack selectivity and are multikinase inhibitors. Moreover, their therapeutic effectiveness may be related to the simultaneous inhibition of more than a single target. Although most of these drugs are used for the treatment of neoplastic disorders, we can expect advances in clinical efficacy and subsequent approval of new drugs targeting additional protein kinases as well as illnesses such as hypertension, Parkinson's disease, and autoimmune diseases [189].

Conflict of interest

The author is unaware of any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this review.

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