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Designing bisubstrate analog inhibitors for protein kinases

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Abstract

Protein kinases play critical roles in signal transduction pathways by transmitting extracellular signals across the cell membrane to distant locations in the cytoplasm and the nucleus. The development of protein kinase inhibitors has been hindered by the broad overlapping substrate specificities exhibited by these enzymes. The design of bisubstrate analog inhibitors could provide for the enhancement of specificity and potency in protein kinase inhibition. Bisubstrate analog inhibitors form a special group of protein kinase inhibitors that mimic two natural substrates/ligands and that simultaneously associate with two regions of given kinases. Most bisubstrate analogs have been designed to mimic the phosphate donor (ATP) and the acceptor components (Ser-, Thr-, or Tyr-containing peptides). Recent studies have emphasized the importance of maintaining a specific distance between these two components to achieve potent inhibition. In this review, we present a discussion of the methods for designing protein kinase inhibitors by mechanism-based approaches. Emphasis is given to bivalent approaches, with an interpretation of what has been learned from more and less successful examples. Future challenges in this area are also highlighted.

Keywords: Protein kinases; Src; Insulin receptor kinase; Bisubstrate inhibitor; Mechanism; Transition state

Abbreviations: AdoC, adenosine-5′-carboxylic acid; CK, casein kinase; CDPK-1, Ca^{2+}-dependent protein kinase 1; CT, C-terminal; EGFR, epidermal growth factor receptor, (F_5)Phe, pentafluorophenylalanine; GABA, γ-aminobutyric acid; IRK, insulin receptor kinase; NT, N-terminal; PKA, protein kinase A; PKC, protein kinase C; PTK; protein tyrosine kinase; RTK, receptor tyrosine kinase; SH, Src homology.

Contents

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1. Introduction

The protein kinase superfamily is one of the largest, as ~2% of eukaryotic genes encode them (Hunter, 1987, 2000). Based on the sequencing of the human genome, ~600 protein kinases are predicted. Protein kinases are enzymes that catalyze phosphorylation of Tyr, Thr, and Ser residues in many proteins by the transfer of the γ-phosphoryl group from ATP. These enzymes are critical components of the cellular signal transduction machinery. Enhanced protein tyrosine kinase (PTK) activity has been directly associated with cell transformation and carcinogenesis (Biscardi et al., 1999; Levitzki, 1999). The role of protein kinases in cancer, inflammation, and cardiovascular diseases provides a driving force for developing inhibitors as therapeutic modulators of pathologic signaling activity.

There are specific binding sites for both protein and ATP in the active site of protein kinases. Both ATP and protein are present in the active site simultaneously, and the transfer of the phosphoryl group takes place directly between these two substrates, without the involvement of a covalent intermediate. In considering the design of bisubstrate analog inhibitors of a protein kinase, it is likely to be important to understand the structural and mechanistic features of the particular target kinase.

1.1. General structural features of protein kinases

The three-dimensional structures of > 20 individual protein kinases have been determined by X-ray crystallography. Discussing the details of the structural features of all protein kinases is beyond the scope of this review. The structural features of several tyrosine kinases are briefly discussed here to provide a framework for considering the protein kinase superfamily and specific aspects of this review. PTKs are subdivided into cytoplasmic nonreceptor tyrosine kinases (non-RTKs) (such as Src), which can be regulated by different mechanisms, and RTKs [such as insulin receptor kinase (IRK)], which are transmembrane cell surface proteins that are activated by the binding of extracellular ligands (such as insulin and many growth factors) and that transduce signals to the cytoplasm by autophosphorylation and phosphorylation of downstream signaling proteins (Hubbard & Till, 2000).

Src kinases are potentially important drug targets, and can serve as an example for the interesting structure and function that protein kinases possess. They are made up of five components/domains: *N*-terminal (NT), Src homology (SH)3, SH2, kinase [catalytic, including NT and C-terminal (CT) lobes], and CT noncatalytic domain (see Fig. 1). The kinase domain is conserved in all protein Ser/Thr and Tyr kinases, is responsible for catalytic activity, and contributes to a varying degree to protein substrate specificity. The conserved ~260 amino acid protein kinase domain architecture folds into a two-lobe structure (Taylor & Radzio-Andzelm, 1994). The kinase domain possesses a smaller NT lobe that is primarily associated with ATP binding and a larger CT lobe that is thought to be primarily associated with peptide/protein substrate binding (Fig. 1). SH3 and SH2 are protein-protein interaction domains that have both adaptor and regulatory functions. SH3 domains are ~60 amino acids in length, and coordinate proline-rich regions that are present in a variety of signaling molecules. SH2 domains are composed of ~100 amino acid residues, and have evolved to recognize and bind to tyrosyl-phosphorylated sequences on proteins (Xu, W. et al., 1999).

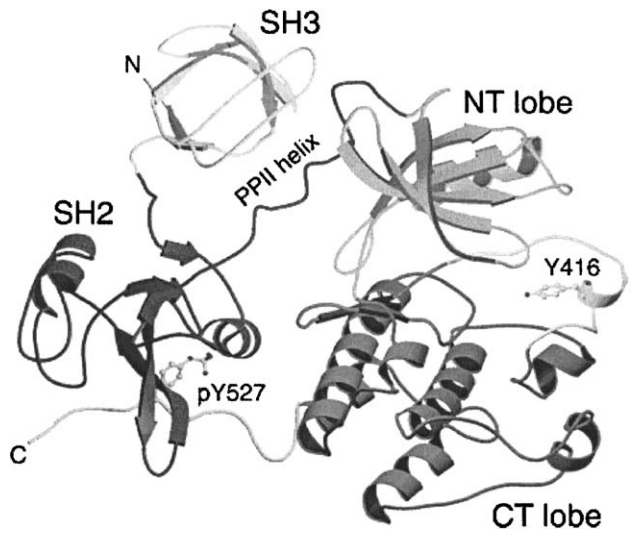


Fig. 1. Structural domains of Src (Xu, W. et al., 1999).

High-affinity monovalent ligands targeting the substrate-binding site (CT lobe), the nucleotide-binding site (NT lobe), and SH2 domain have been developed (Songyang et al., 1993; Showalter & Kraker, 1997; Druker & Lydon, 2000; Vu, 2000). Protein kinases can be inhibited by compounds competing with ATP for its binding pocket (NT lobe) on the enzyme molecule. The design of inhibitors selective for protein kinases based on nucleotide binding presents a significant challenge, due to the presence of a large number of protein kinases that show a conserved nucleotide pocket. There have been some significant successes in designing kinase inhibitors directed toward the nucleotide-binding site (Toledo et al., 1999; Traxler, 1998; Lawrence & Niu, 1998; Topaly et al., 2001; Thiesing et al., 2000). These inhibitors include maleimides, phthalimides, pyrazolopyrimidines, quinazolines, oxindoles, pyridopyrimidines, isoquinoline, and several other classes of compounds (Toledo et al., 1999; Traxler, 1998; Lawrence & Niu, 1998). Some of these compounds have shown potent inhibitory action against particular kinases (K_i in nanomolar-picomolar range). Some ATP-competitive inhibitors can even distinguish between closely related tyrosine kinases (for example, between Src kinases) (Showalter & Kraker, 1997; Druker & Lydon, 2000), and so, are very promising drug leads. Recently, Gleevec (ST1571, 1, Fig. 2), which is an Abl Tyr kinase inhibitor, was approved by the United States Food and Drug Administration for the treat-

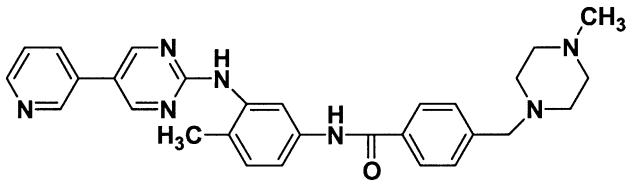


Fig. 2. Chemical structure of Gleevec (ST1571) (**1**), a new Abl tyrosine kinase inhibitor recently approved by the United States Food and Drug Administration that is highly effective in treating the early stages of chronic myeloid leukemia (Topaly et al., 2001; Thiesing et al., 2000).

ment of chronic myelogenous leukemia (Topaly et al., 2001; Thiesing et al., 2000), but remissions induced in advanced phases tend to be relatively short-lived, an observation that suggests the development of de novo resistance to the drug. Gorre et al. (2001) showed that "acquired" resistance to STI-571 is often associated with reactivation of the tyrosine kinase activity of the BCR-ABL oncoprotein.

Inhibitors competing with the peptide or protein substrate for their binding site have also been described (Kemp et al., 1991; Lawrence & Niu, 1998). This class of compounds has mostly been comprised of peptides containing the substrate consensus motif that makes it possible to generate greater specificity. In general, substrate peptide-based inhibitors, in spite of their potential for selectivity, have exhibited relatively weak inhibitory performance (K_i in the high micromolar range).

SH2-containing targets have been the subject of intense research by a number of researchers for therapeutic intervention (for a review, see Vu, 2000). For example, tetrapeptide phosphotyrosine-glutamic acid-glutamic acid-isoleucine (pTyr-Glu-Glu-Ile, pYEEI) has high affinity (K_d, 100 nM) for the SH2 domain of Src tyrosine kinase (Songyang et al., 1993).

1.2. General mechanistic features of protein kinases

As mentioned in Section 1, the catalytic mechanism of protein kinases involves ternary complex formation, consisting of a protein kinase, a protein substrate, and MgATP (Ho et al., 1988; Cole et al., 1994). The direct transfer of the phosphoryl group from ATP to the protein substrate may occur, in the extreme, by two different mechanisms (Fig. 3). First, a fully dissociative mechanism, where the bond between the attacked phosphorus and ADP (leaving group) is largely broken in the transition state, while the bond between the nucleophile (Tyr, Thr, or Ser) and phosphorus has not yet formed. Similar to an S_N1 reaction in organic chemistry, the nucleophilicity of the attacking hydroxyl is not important. Second, an associative mechanism with a transition state containing a significant amount of bond formation between the nucleophile and phosphorus, with relatively little bond breakage between the phosphorus and the leaving group. Dissociative transition states are generally accepted for the corresponding nonenzymatic phosphate monoester phosphoryl transfer reactions (for a dissenting view, see Aqvist et al., 1999), but have been controversial for enzyme-catalyzed reactions (Admiraal & Herschlag, 2000; Mildvan, 1997).

In principle, the two mechanisms can be distinguished by use of kinetic thio effect measurements, linear free energy plots, and kinetic isotope effect measurements. Two of these approaches have been applied to PTK CT Src kinase and the IRK, and have supported a dissociative transition state for these enzymes and, by extension, other protein kinases (Kim & Cole, 1998; Ablooglu et al., 2000). In addition, the paradoxical observation was made that the neutral phenol is more enzymatically reactive than the phenoxide anion. These results also indirectly support a dissociative transition state, because they imply that nucleophilic reactivity is less important than hydrogen bonding pattern within the active site.

While it is impossible to know how precisely these transition state features would correspond to geometric requirements, an interesting proposal has been put forward by Mildvan (1997) in this regard. The argument is that for a free and clear metaphosphate to form, this species must be at least 3.3 Å (the sum of the van der Waals radii) away from the entering nucleophile and 3.3 Å away from the departing

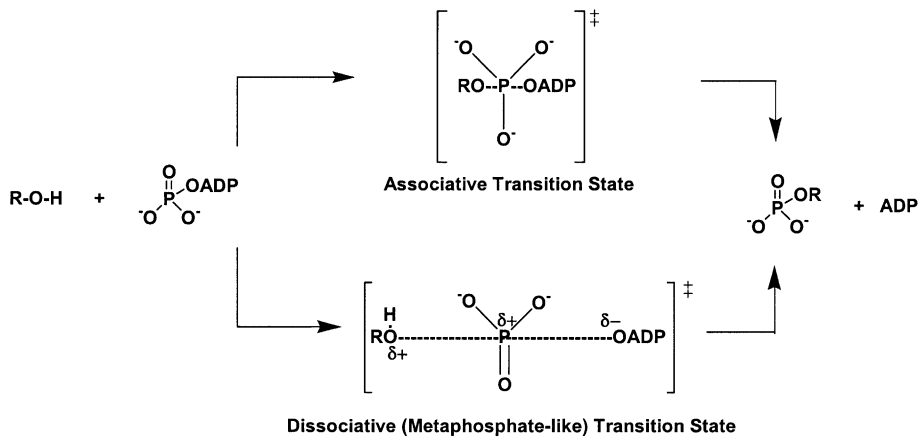


Fig. 3. Associative versus dissociative transition states for phosphoryl transfer (Aqvist et al., 1999).

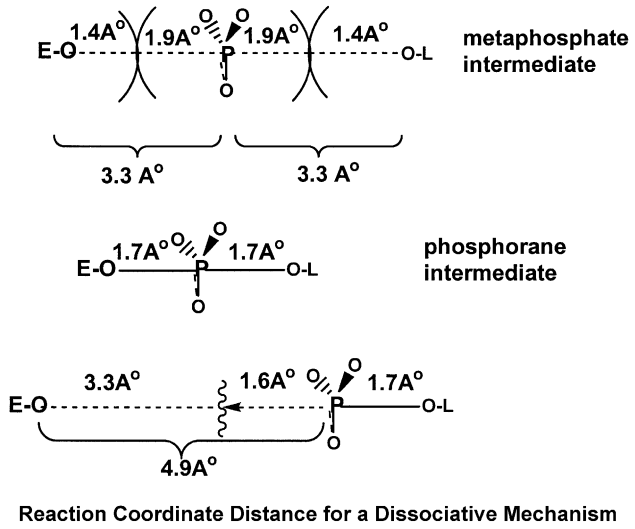


Fig. 4. Geometric requirements for associative and dissociative transition states. E-O, entering-oxygen; O-L, oxygen-leaving group. (Mildvan, 1997).

leaving group (see Fig. 4). In contrast, for an associative transition state, the corresponding distances could be smaller than 2 Å. If one further assumes that in the phosphoryl transfer, the larger entering group and leaving group are relatively fixed and the metaphosphate-like species undergoes most of the movement, a (reaction coordinate) distance > 4.9 Å is necessary to allow for a free metaphosphate intermediate. This so-called reaction coordinate distance probably would correspond to the ground-state ternary complex that precedes the dissociative transition state. A reaction coordinate distance of $\leq$ 3.3 Å in a transition state indicates greater associative character and compression (Mildvan, 1997) because new bond formation to the entering atom can occur before old bond breakage from the leaving group is complete (Fig. 4).

In attempting to design kinase inhibitors, consideration of these potential geometric constraints for the dissociative (as opposed to the associative) reaction mechanisms may be important. Bisubstrate analog inhibitors for protein kinases may be designed to imitate the binding determinants in such a complex (see Section 2).

2. Bisubstrate analog inhibitors (general)

Designing bisubstrate analog inhibitors is an established and effective strategy to enhance the potency and specificity in enzyme inhibition for many enzymes (Radzicka & Wolfenden, 1995; Broom, 1989). A very high order of enzyme specificity may be expected, since the combination of two substrates required by the target enzyme into a single molecule makes it likely that neither component will be recognized by other enzymes using either substrate (Broom, 1989). A greater selectivity may arise if one of the substrates demonstrates specific binding to its binding pocket. From a thermodynamic point of view, a large energetic advantage in binding is expected for a bisubstrate analog inhibitor that incorporates binding motifs of two substrates within the same molecule, as compared with the binding properties of analogs of two substrates measured separately (Frick et al., 1989). For example, the free energy for the bisubstrate analog inhibitor theoretically could be equal to the sum of the free energies for each component plus an additional energetic factor that might be worth up to 11 kcal/mol (in entropy or enthalpy) (Page & Jencks, 1971; Bruice & Benkovic, 2000).

In practice, the most potent bisubstrate analog inhibitors tend to have affinities that are equal or slightly greater than the sum of the binding energy for each substrate component. The initial bisubstrate analog inhibitor designed and synthesized, *N*-(phosphonacetyl)-L-aspartate, a potent and selective inhibitor of aspartate transcarbamylase, showed an affinity that was approximately the sum of the binding energy of aspartate and carbamyl phosphate as single components (Collins & Stark, 1971). Subsequently, there have been many other examples too numerous to discuss in detail here (Radzicka & Wolfenden, 1995; Liu et al., 2000). One notable example in the kinase field, however, is P^1, P^5-bis(5′-adenosyl)pentaphosphate, a potent inhibitor of aden-ylate kinase (Lienhard & Secemski, 1973). It should be noted that P^1,P^5-bis(5′-adenosyl)pentaphosphate has five phosphate groups, one more than the sum of the number of phosphates in the two substrates (ATP and AMP, total of four phosphates). This increased number may be important for the optimized reaction coordinate distance.

Not all bisubstrate analog inhibitors are strong enzyme inhibitors. For example, the bisubstrate analog inhibitors prepared to block hexokinase P^1-(5-adenosyl)-P^3-(6-glucosyl)triphosphate and P^1-(5-adenosyl)-P^4-(6-glucosyl)tetraphosphate are only weak inhibitors of this enzyme, with apparent affinities lower than either substrate ATP or glucose (Danenberg & Danenberg, 1977). Thus, consideration of the reaction mechanism and complementarity of the linker may be essential in developing potent inhibitory compounds.

2.1. An ideal bisubstrate analog inhibitor for protein kinases

In general, a bisubstrate analog inhibitor for protein kinases should have the following characteristics for an optimal effect. The inhibitor should (1) inhibit the binding of both natural substrates to their binding pockets; (2) be designed based on the geometry and orientation of natural substrates and their interface with their binding sites in protein kinases; (3) be specific against one particular protein kinase (this is especially important, due to the presence of overlapping natural substrates for a very large number of protein kinases); and (4) show good bioavailability and low toxicity.

2.2. Different strategies in designing bisubstrate analog inhibitors for protein kinases

During the last 15 years, many different approaches have been examined for the possibility of using bisubstrate analog inhibitors for protein kinases. These strategies fall into four categories: (1) sulfonamides and sulfonylbenzoyl derivatives, (2) carboxylic acid derivatives, (3) dipeptidyl derivatives, and (4) phosphodiester derivatives. The criteria noted in the previous section will be considered in evaluating the compounds in each category. None of the strategies have all the characteristics for an optimal bisubstrate analog inhibitor for protein kinases, but substantial progress has been made in this direction.

2.2.1. Sulfonamides and sulfonylbenzoyl derivatives

Kruse et al. (1988) synthesized and tested bisubstrate inhibitors of tyrosine-specific protein kinases. One of the substrates, ATP, was mimicked by the known kinase inhibitor 5′-[4-(fluorosulfonyl)benzoyl]adenosine (see Compound **2**, Fig. 5; IC_{50}, 150 μM). The carbonyl and the sulfonyl groups assumed to mimic the α- and γ-phosphates of ATP, while the fluorosulfonyl functionality was used as an attachment site for tyrosine mimics such as aminoalkyl-substituted phenyl to probe the distance between the ATP and tyrosine-binding sites and to mimic the transition state (see Compound **3**, Fig. 5). The NH group was considered to be a mimic of the phenolic hydroxyl of the tyrosine substrate. The resulting bisubstrate inhibitors were tested against $p60^{v\text{-}abl}$, the tyrosine kinase encoded by the transforming gene (*v-abl*) of the Abelson murine leukemia virus. These inhibitors displayed moderately potent activity (IC_{50} values ranging from 19 μM for n = 0 to 56 μM for n = 3). These bisubstrate analog inhibitors bind primarily through the adenosine moiety common to all the inhibitors due to the absence of large effects produced by modification of the tyrosine mimics. The sulfonamides displayed similar inhibition patterns for the Ser kinase/phosphorylase kinase, at comparable concentrations, supporting the conclusion that the compounds bind primarily at the ATP site and lack specificity. Varying the chain length between the NH and aromatic ring (n = 0–3) had little effect on the inhibitory potency of the compounds. These results also indicate that the tyrosine substrate moiety is not efficiently mimicked in these compounds, which behave primarily as ATP-binding site inhibitors.

A related sulfonamide approach has also been used to develop inhibitors for protein kinase A (PKA) (Ricouart et al., 1991). This class of Ser/Thr protein kinase inhibitors was designed to bind simultaneously to the ATP and protein substrate-binding sites of PKA. In earlier work, it had been shown that peptides containing the sequence of the endogenous PKA inhibitors had a greater inhibitory effect compared with peptides containing a minimal substrate consensus motif (Walsh & Glass, 1991). Ricouart et al. (1991) hypothesized that basic residues such as Arg proximal to the phosphorylated Ser (or Thr) are important for the substrate specificity of PKA. They exploited this tendency by coupling isoquinoline or naphthalene sulfonic acids (as ATP mimics) with peptides containing oligoarginines. For the first series of compounds (Fig. 5), a flexible linker consisting of two β-alanine residues was installed between two moieties. Some of these compounds inhibited PKA and protein kinase C (PKC) at submicromolar concentrations (IC_{50} ranged from 0.3 to > 75 μM). It was found that the most potent compound (**4**) consisted of a cluster of 6 Arg residues (K_i of 8 μM for PKC and 0.17 μM for PKA) that was a competitive inhibitor toward ATP, but not toward peptidicsubstrates. For the second series of compounds, the two moieties were covalently bound by a - $NH(CH_2)_2NH$

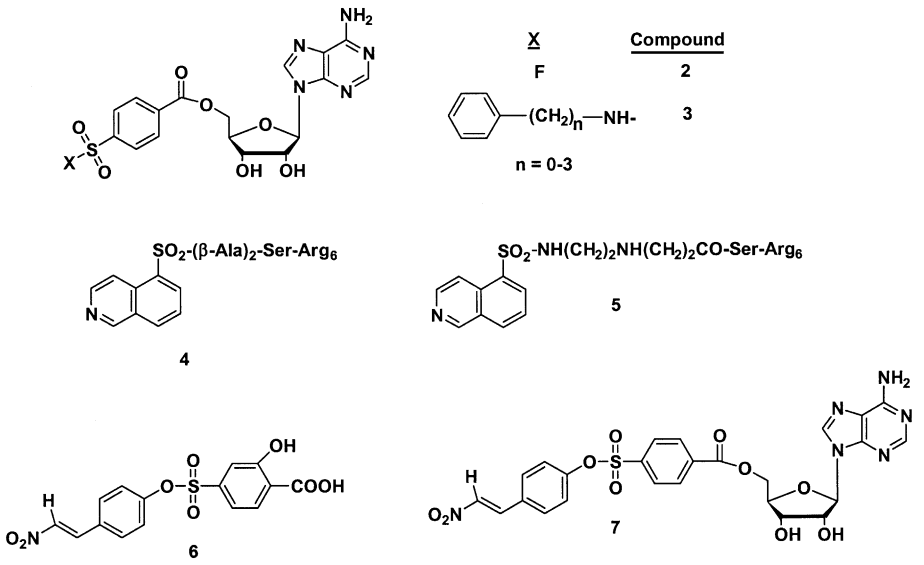


Fig. 5. Sulfonamides and sulfobenzoyl derivatives as bisubstrate analog inhibitors for protein kinases (Kruse et al., 1988; Ricouart et al., 1991; Traxler et al., 1991).

(CH_2)CO- linker, which was suggested to model the distance observed in phosphoglycerate kinase. Compound ***5*** (Fig. 5) consisted of isoquinoline-5-sulfonamide, as ATP mimic, and Ser-Arg_6, as peptidic moiety, and had a K_i of 0.1 μM toward PKC and 0.004 μM toward PKA. Kinetic studies indicated that Compound **5** was a competitive inhibitor toward ATP, but not competitive against the peptide substrate, even at ATP concentrations as high as 50 μM. Since PKA is known to show a preference for ATP binding prior to the peptide substrate, the lack of a pattern of competitive inhibition by **5** against the peptide substrate does not rule out dual occupancy of the two sites.

A series of sulfonylbenzoyl-nitrostyrene derivatives (Fig. 5) were synthesized by Traxler et al. (1991) as bisubstrate type inhibitors of the epidermal growth factor receptor (EGFR) tyrosine protein kinase that may mimic both ATP, tyrosine, and the transition state. The most active compound, **6**, with an IC_{50} value of 0.054 μM for EGFR, also exhibited high selectivity with respect to *v-abl* tyrosine kinases (IC_{50}, 27 μM) and to PKC (IC_{50}, 500 μM). A computer-generated model for the transition state of the γ-phosphoryl transfer from ATP to a tyrosine moiety was used for fitting experiments using the highly potent derivative Compound **6**. The model supported the hypothesis that one of the oxygen atoms of the carboxylic group in **6**, together with the *ortho* hydroxyl group, can be superimposed on the oxygen atoms of the γ- and β-phosphoryl groups of ATP and mimics a diphosphate moiety in the transition state. These groups are involved for coordination with Mg^{2+} in the transition state. Based on this model, it was predicted that the aromatic ring, together with the sulfonyl group of sulfobenzoyl moiety, spans the same distance of γ-phosphate to *O*-Tyr of ∼ 5.3 Å. No kinetic studies were carried out to examine the inhibition pattern for these analogs, and it was not clear how this compound acted as a bisubstrate inhibitor for both the substrate-binding site and the ATP-binding site of the EGFR tyrosine kinase. The addition of an adenosyl moiety to generate Compound **7** resulted in a reduction of potency and selectivity (IC_{50} values of 0.6 μM for EGFR, > 100 μM for *v-abl* tyrosine kinases, and 10 μM to PKC), calling into question the proposed model.

Rossé et al. (1997) reported the synthesis of a series of bisubstrate inhibitors of the EGFR protein kinase by linking small peptides covalently to adenosine. A transition state model was proposed for the transfer of the γ-phosphoryl group of ATP to a tyrosine moiety in a substrate molecule, with a pentacoordinate P(γ) atom and with the α- and β-phosphate groups complexed with two bivalent metal ions (usually Mg^{2+} or Mn^{2+}) and Arg817. Based on the postulated model, a series of bisubstrate inhibitors, consisting of a tri- or tetrapeptide as the protein substrate substitute; sulfobenzoyl derivatives (sulfobenzoyl **8**, 2-hydroxy-4-sulfobenzoyl **9**, benzene-1, 4-disulfonyl **10**, or adipoyl moiety **11**) as the triphosphate mimic or spacer; and adenosine were synthesized (Fig. 6). The sequences Glu-Tyr-Leu and Glu-Tyr-Leu-Arg were derived from a consensus sequence for the phosphorylation site of natural substrates of EGFR and the major autophosphorylation site of the EGFR, respectively (Pearson & Kemp, 1991; Yarden & Ullrich, 1988). The most active compound, **12**, was composed of the tripeptide sequence H-Glu-Tyr-Leu-OBzl, the 2-hydroxy-4-sulfonylbenzoyl moiety, and adenosine (IC_{50}, 33 μM). Removal of the hydroxyl group of the 4-sulfobenzoyl caused a significant decrease in the activity against the EGFR, since no inhibitory effect was observable at

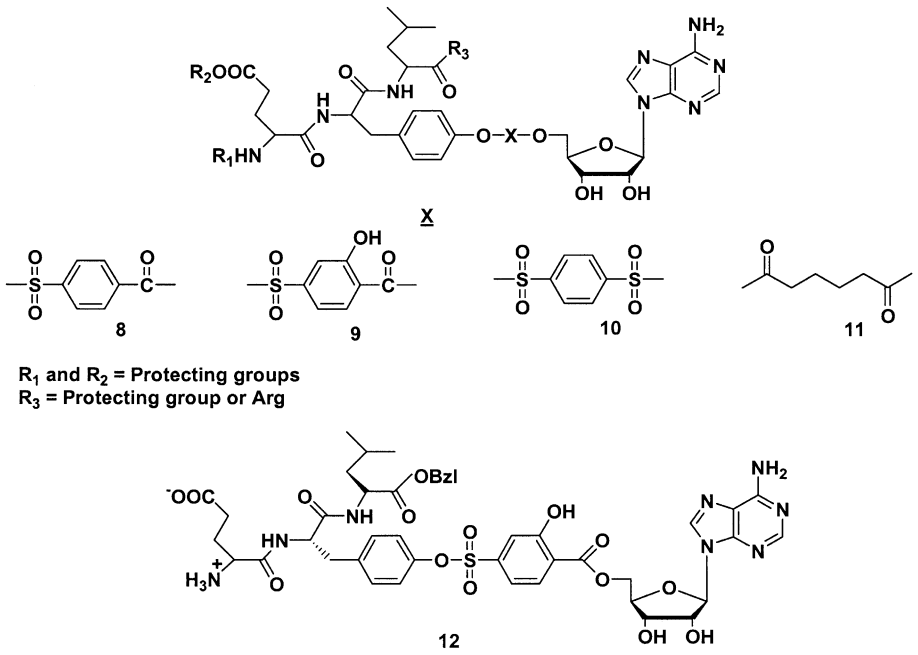


Fig. 6. Possible bisubstrate analog inhibitors of the EGFR (Rossé et al., 1997).

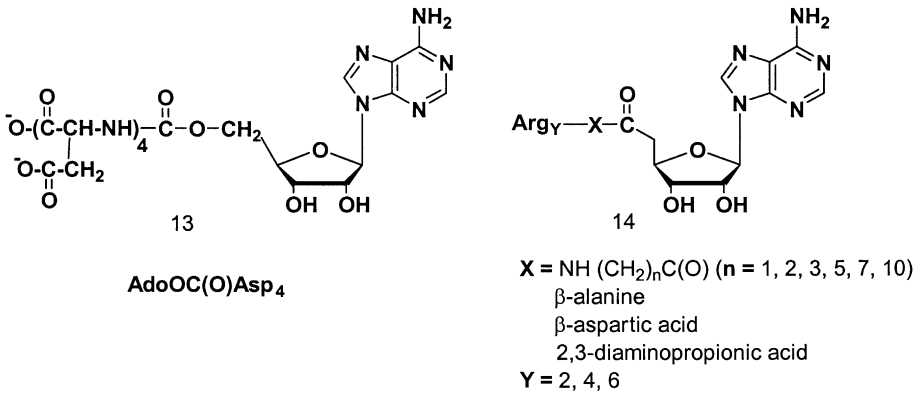


Fig. 7. Proposed bisubstrate analog inhibitors with carboxylic acid moiety (Uri et al., 1994; Pehk & Uri, 1997; Loog et al., 1999).

100 μM. It was assumed that the additional OH group could be important in enhancement of the complexation of bivalent metal ions. Kinetics studies to elucidate the inhibition pattern for Compound **12** have not been reported to date.

2.2.2. Carboxylic acid derivatives

Uri and co-workers (Uri et al., 1994; Pehk & Uri, 1997) prepared a series of peptidyl derivatives of adenosine-5′-carboxylic acid (AdoC) such as the tetra-aspartate analogs (**13**) (see Fig. 7) as potential ligands for P2 purinoceptors. The solid phase synthesis of these analogs involved the attachment of AdoC to a Wang-type polystyrene resin.

The same group (Loog et al., 1999) used a similar strategy for designing bisubstrate analog inhibitors for protein kinases. Based on a strategy related to Ricouart et al. (1991), inhibitors were synthesized by coupling AdoC with a peptide moiety via various linkers, such as β-alanine, β-aspartate, 2,3-diaminopropionic acids, and $NH(CH_2)_nC(O)$ (**14**) (Fig. 7).

Loog et al. (1999) found several potent inhibitors of PKA, PKC, and Ca^{2+}-dependent protein kinase 1 (CDPK-1) among the polyarginine-containing compounds. One of the most potent compounds, $AdoC(Ahx)Arg_6$ (X=Ahx= $NH(CH_2)_5C(O)$, aminohexanoic acid) inhibited PKA, PKC, and CDPK-1 with IC_{50}s of 0.12 μM, 0.27 μM, and 1.2 μM, respectively, but exhibited low activity against casein kinase (CK)1 and CK2 ($IC_{50} > 30$ μM). This selectivity against CK1 and CK2 was expected since these enzymes preferentially process negatively charged peptide sequences (Pinna & Ruzzene, 1996), whereas the substrate preferences of PKA and PKC, as well as CDPK-1, is for positively charged amino acids (Muszynska et al., 1993, Kennelly & Krebs, 1991). Recently, the same group used similar bisubstrate analog inhibitors linking adenosine and polyarginine for affinity chromatography of protein kinases (Loog et al., 2000). The affinity and selectivity of the inhibitors were modified by selecting proper peptide moieties. Two affinity ligands, $Ado\text{-}Aoc\text{-}Arg_4\text{-}Lys$ (ligand A) and $AdoC\text{-}Aoc\text{-}Arg_4\text{-}MH(CH_2)_6NH_2$ (ligand B) (Aoc, amino-octanoic acid), were synthesized and tested for the ability to chromatographically retain PKA. The bisubstrate ligands were conjugated to the epoxy-activated Sepharose via the side chain amino group of their termini. These ligands bind selectively to "basophilic" protein kinases. Homogenous protein kinase was eluted with MgATP, as well as L-arginine in a single purification step (Loog et al., 2000). Protein kinase with an acidic specificity determinant (CK2) did not bind to the column (Loog et al., 2000). This approach may be useful for the isolation and extraction of PKA from biological materials.

2.2.3. Dipeptidyl and N-acylated peptide derivatives

Profit et al. (1999, 2001) have investigated bivalent peptidyl analog inhibitors that simultaneously bind to the active site and the SH2 domain of the Src kinase (see Fig. 8). Using this approach, they probed the distance between the SH2 and active site regions of the Src PTK, and evaluated the topological and spatial relationships of these regions. These bivalent analogs consisted of two linked peptides, the kinase active-site directed (-EELL(F_5)Phe-, where (F_5)Phe is pentafluorophenylalanine), and SH2 directed (-pYEEIE-), connected by a variable number of γ-aminobutyric acid units (GABA), $(Abu)_n$. The active-site targeted moiety, -EELL-(F_5)Phe- (IC_{50}, 1.59 mM), was selected based on a synthetic library study (Niu & Lawrence, 1997). The SH2-targeted moiety, -pYEEI-, was derived from the SH2 recognition motif present in the hamster polyoma virus middle T antigen (Songyang et al., 1993). Peptides based upon this sequence exhibit high affinity for the SH2 domains of Src kinase family members (K_d values in the 1–5 μM range) (Lee & Lawrence, 1999). The general structure of these bivalent analogs was

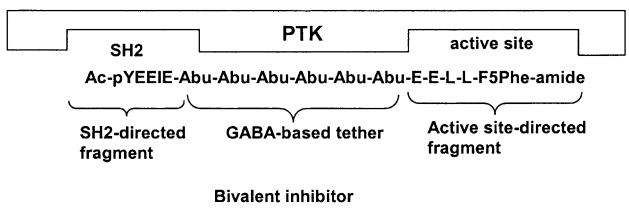


Fig. 8. General structures of Src kinase-targeted bivalent ligands (Profit et al., 1999, 2001).

Ac-pYEEIE-$(Abu)_n$-EELL-(F_5)Phe-amide, where *n* varied, in multiples of 2, from 4 to 12. The modest affinity of the active site-targeted peptide indicates that the SH2-targeted sequence is responsible for the high-affinity component of the bisubstrate analog inhibitor. The compounds were evaluated along with the active site-directed control peptide, Ac-EELL-(F_5)Phe-amide (**15**), for their ability to inhibit c-Src-catalyzed phosphoryl transfer (Profit et al., 1999). Peptide Ac-pYEEIE-$(Abu)_6$-EELL-(F_5)Phe-amide (**16**) (Fig. 9), with an IC_{50} of 35 μM, exhibited a 45-fold enhanced inhibitory potency for the Src kinase relative to control peptide Ac-EELL-(F_5)Phe-amide (**15**), with an IC_{50} of 1590 μM against Src. Another control peptide, Ac-$(Abu)_8$-EELL-(F_5)Phe-amide (**17**), that lacks the SH2 recognition sequence was a much poorer inhibitor (IC_{50}, 800 μM). Furthermore, saturation of the SH2 domain of Src with 300 μM of Ac-pYEEI-amide (**18**) reduced the potency of **16** (IC_{50}, 240 μM). Both experiments indicate the significant contribution of the SH2 recognition sequence in total activity, and serve as good evidence for the binding of **16** to both binding sites (Profit et al., 1999). Another related compound with longer linker length, Ac-pYEEEIE-$(Abu)_8$-EELL-(F_5)Phe-amide (**19**), was discovered by the same group, and it exhibited an IC_{50} of 18.5 μM, suggesting that the Tyr(P)- and (F_5)Phe-binding sites are separated by > 40 Å (Profit et al., 2001).

In another study, coumarin-pYEEIE-$(Abu)_8$-EELL-(F_5)Phe-amide (**20**) (IC_{50}, 6.9 μM) and (biotin-aminohexanoic acid)-pYEEIE-$Abu)_6$-EELLY-amide (**21**) were used to evaluate the inhibition pattern with an avidin-impregnated membrane and Src. Peptide **21** exhibited a K_m of 47 μM and a V_{max} of 130 nmol/min-mg, while **20** displayed an IC_{50} of 19 μM with this substrate, and was a competitive inhibitor versus variable peptide substrate **19**, with a K_i of 9 μM (Fig. 9) (Profit et al., 1999).

Recently, a more potent bisubstrate analog inhibitor was discovered by Profit et al. (2001) in an antiparallel approach using active site peptide at the C-terminus linked by GABA to SH2-targeted peptide at the N-terminus of the compound. The bisubstrate compound **22**, acetyl-EELL-(F_5)Phe-$(Abu)_3$-pYEEIE-amide (IC_{50}, 13 μM), exhibited an ~ 120-fold greater inhibitory potency than the simple monovalent active site-directed species acetyl-EELL-(F_5)Phe-amide (**15**) (IC_{50}, 1590 μM). The linker for Compound **22** is shorter than that of Compounds **19** and **16**, and since **22** exhibited greater potency, it is implied that the spatial arrangement for **22** is preferred (Fig. 9). More studies are required to explain the binding mechanism for the peptide moieties in the bivalent inhibitors. It should also be mentioned that for each of these bivalent inhibitors, the apparent active site affinity of the kinase-binding sequence is less

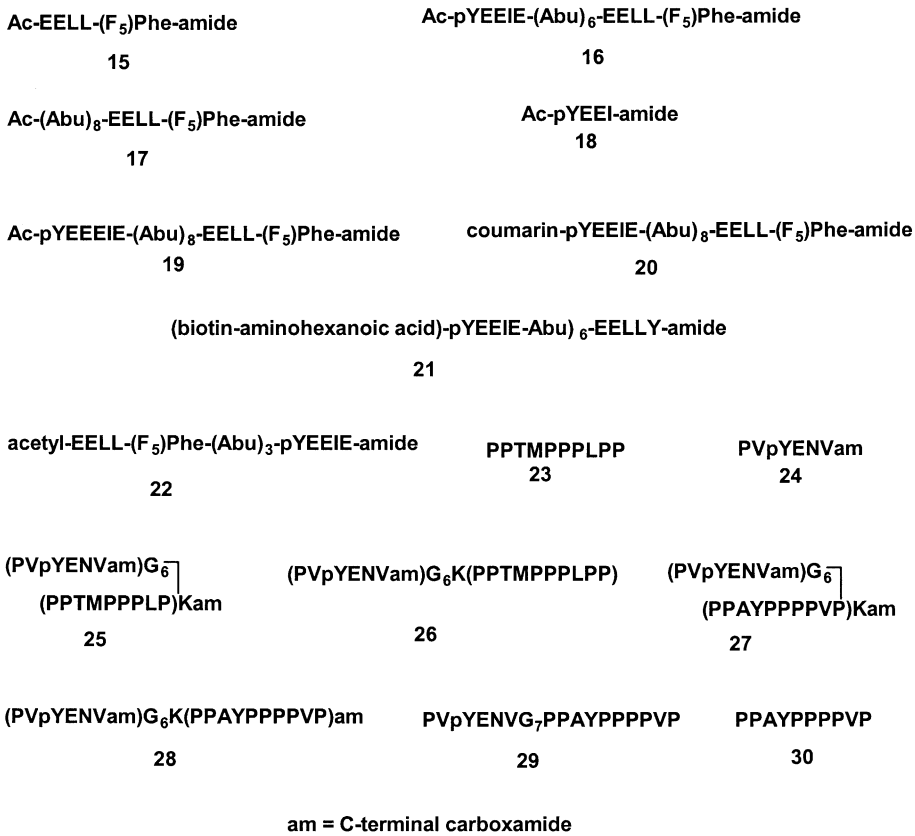


Fig. 9. Monopeptidyl, dipeptidyl, and *N*-acylated peptide derivatives as bisubstrate analog inhibitors for protein kinases (Profit et al., 1999, 2001; Cowburn et al., 1995; Zhu et al., 1994; Musacchio et al., 1994; Xu, Q. et al., 1999).

than the affinity of the SH2-binding sequence, implying that fractional occupancy of the kinase-binding site is relatively low. This suggests that optimization of the tether is possible so that one can start to approach the more desirable goal of additive-binding energies for each of the interactions.

As discussed in Section 1.1, several PTKs contain adjacent SH3 and SH2 domains that are important for their function in signal transduction. A series of "consolidated ligands" were prepared by Cowburn and co-workers (Cowburn et al., 1995; Xu, Q. et al., 1999) with different peptides targeting SH3 and SH2 domains (Fig. 9). As described in Section 1.1, SH3 domains recognize sequences with multiple proline residues and SH2 domains bind to phosphotyrosine containing sequences. A crystal structure of Abl SH3 and PPTMPPPLPP (**23**) has been reported (Musacchio et al., 1994). Ligand PVpYENVamide (**24**) binding to the SH2 domain has been modeled (Zhu et al., 1994). When tethered, these analogs were assumed to bind simultaneously to the SH2 and SH3 domains of the Abelson PTK (Abl) with enhanced affinity. Bivalent ligands exhibited enhanced affin ity and specificity compared with monovalent equiva ents. Different consolidated ligands were prepared based on these structural sequences. Branched peptides, **25** and **27** (K_d values of 0.49 and 0.25 μM), exhibited an increase in affinity of approximately two orders of magnitude compared with unbranched equivalents **26** and **28** (K_d values of 18.0 and 22.1 μM), respectively. Compound **27** showed an order of magnitude increase in affinity (K_d, 0.25 μM) compared with the most strongly bound single ligand to the equivalent consolidated ligand **24** (K_d, 2.4 μM). Recently, these investigators also prepared subfamilies of these ligands that contain different relative orientations of the SH2- and SH3-directed sequences (Xu, Q. et al., 1999). These analogs exhibited enhanced affinity to the regulatory apparatus (SH2 and SH3 domains) of Abl, but their affinities were found to vary with binding portion topologies and linker lengths. From four types of ligands representing all possible relative orientations of ligands, the tightest binding ligand was $PV_pYENVG_7PPAYPPPPVP$ (**29**; IC_{50}, 0.19 μM), (SH2-directed ligand)-tether-(SH3-directed ligand), where the tether is composed of 7 Gly residues, as compared with the most strongly bound monovalent equivalent (**24**; K_d, 2.4 μM). Peptides $PV_pYENVamide$ (**24**) and PPAYPPPPVP (**30**), joined by oligoglycyl linkers, were recognized by SH2 and SH3 domains, respectively (Xu, Q. et al., 1999).

2.2.4. Phosphodiester derivatives

An attempt was made by Medzihradszky et al. (1994) to design a more accurate model of the bisubstrate complex by direct coupling of the Ser residue of a specific PKA substrate, kemptide, to adenosine nucleotides via a phosphodiester bond. The bisubstrate analog inhibitors containing ADP, ATP, and AP_4 (Fig. 10) were tested as inhibitors of the catalytic subunit of PKA. The peptide moiety was based on the well-known substrate peptide (kemptide, LRRASLG) of PKA (Kemp et al., 1977). These compounds revealed

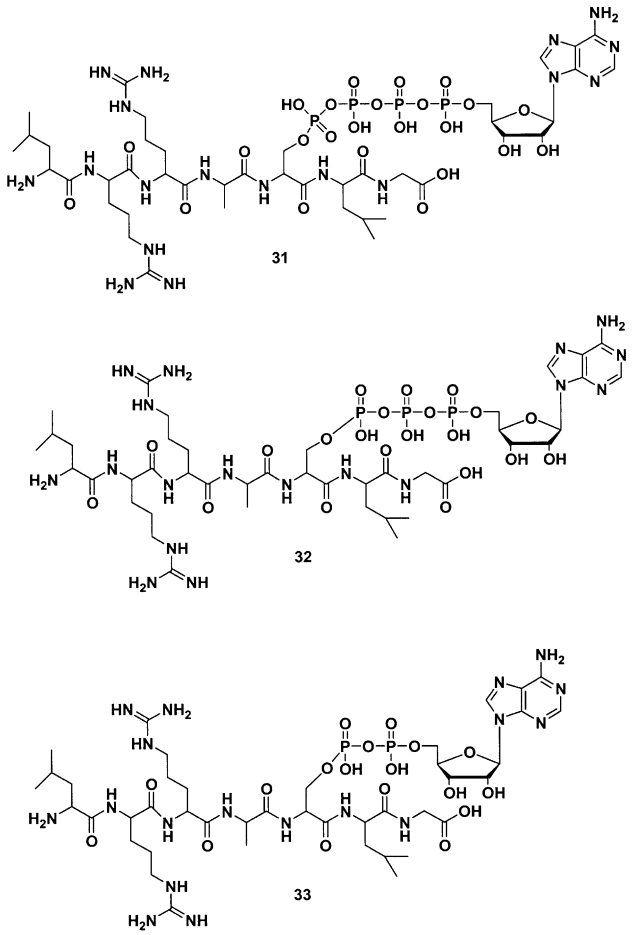


Fig. 10. Structures of kemptide-adenosine 5′-phosphates (Medzihradszky et al., 1994).

only moderate inhibitory effects. The adenosine 5′-tetraphosphate tetrapeptide was the best inhibitor of the series against PKA (**31**, kemptide-AP_4) (IC_{50}, 68 μM). For comparison, the measured K_m values for kemptide and ATP were 15.3 and 9.6 μM, respectively, in their assays. The inhibitory potency was found to decrease with shorter phosphate chains, yielding IC_{50} values of 226 and 935 μM for kemptide-ATP (**32**) and kemptide-ADP (**33**), respectively. All three inhibitors in this series acted competitively with respect to ATP, but not with respect to the peptide substrate (Medzihradszky et al., 1994). The distance between the γ-phosphate and the attacking hydroxyl of Ser in Compound **32** is ∼ 1.7 Å, which is expected to be more appropriate for a fully associative phosphoryl transfer reaction mechanism.

Several other previous mechanism-based approaches to design protein kinase inhibitors (Rossé et al., 1997; Yuan et al., 1990; Ablooglu et al., 2000; Cushman et al., 1990) have led to relatively weak inhibitors. Recently, a new approach to protein kinase inhibition was established using a bisubstrate analog inhibitor, based in part on mechanistic and structural features of a predicted dissociative transition state for tyrosine kinases (Parang et al., 2001) (Fig. 11).

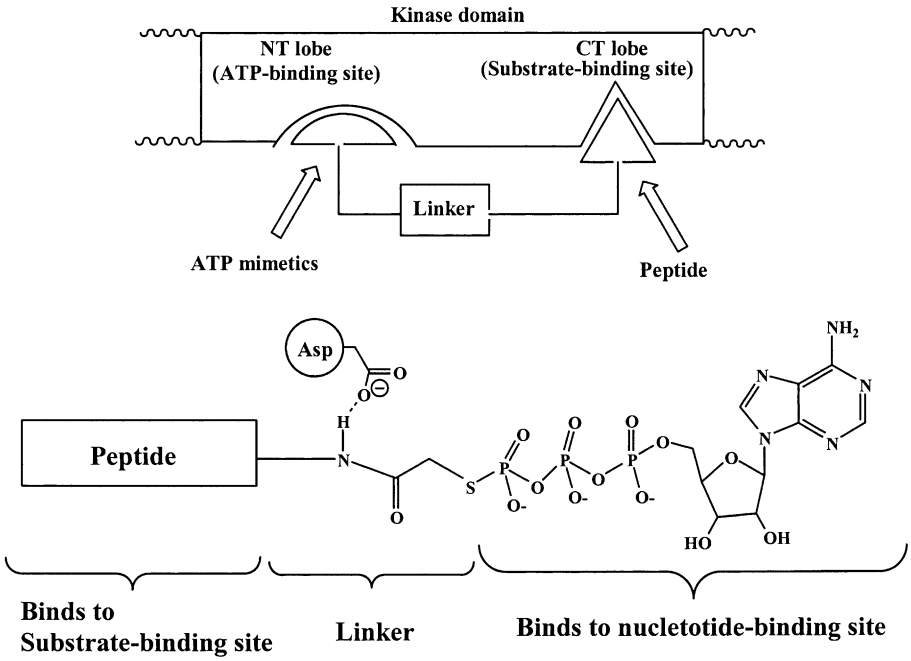


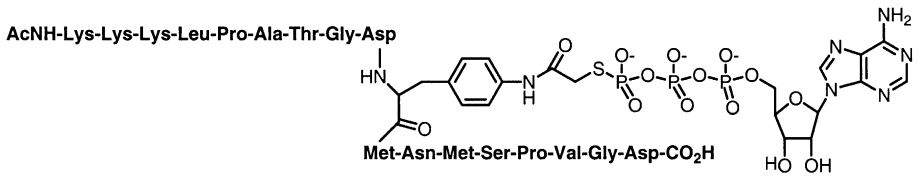
Fig. 11. Designed bisubstrate analog inhibitor for IRK (Parang et al., 2001).

Compound **34** (Fig. 12) was a potent and selective peptide-ATP bisubstrate inhibitor that simultaneously binds to the NT and CT lobes of the IRK.

IRK is an RTK, and its β-subunit located intracellularly contains the tyrosine kinase domain (Schlessinger, 2000). A high-resolution structure of this enzyme in complex with an ATP analog (AMP-PNP) and a peptide substrate (Hubbard, 1997) has been determined. The crystal structure and solution studies suggest a dissociative transition state for this enzyme (Ablooglu et al., 2000). Many efficient peptide substrates (including IRS727) for IRK have been well characterized kinetically (Ablooglu et al., 2000), but no potent inhibitors had been reported for this important signaling enzyme.

The peptide portion of the bisubstrate is modeled based on the known insulin RTK substrate (sequence Asp-Tyr-Met-Asn-Met) that is derived from a phosphorylation site on insulin receptor substrate-1 (Songyang et al., 1995). The protected substrate-binding site-directed peptide, in which the synthon for the single Tyr equivalent of IRS727 was replaced with commercially available nitrophenylalanine, was prepared on resin, utilizing a standard Fmoc solid-phase synthesis protocol. The N-terminus was protected with an acetyl group by the action of acetic anhydride. After reduction of the nitro to an amino group with $SnCl_2$, the peptide was subjected to bromoacetylation. The bromoacetylated peptide was cleaved from the resin, deblocked, and then purified by preparative reversed-phase HPLC.

ATPγS was used as the ATP mimic to take advantage of the known nucleophilic chemoselectivity of the γ-sulfur. This was predicted to result in a 5–5.7 Å spacer between the attacked phosphorus and the nucleophilic anilino nitrogen



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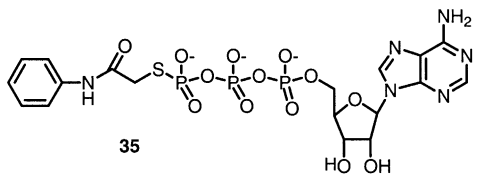

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Fig. 12. Designed protein kinase bisubstrate analog inhibitors (Parang et al., 2001).

(analogous to the tyrosine hydroxyl) (Fig. 11), compatible with the Mildvan prediction for a dissociative transition state discussed in Section 1.2. The bromoacetylated peptide was reacted with adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) in aqueous solution at pH 7, and the peptide-ATP conjugate was purified by reverse-phase HPLC and characterized by electrospray mass spectrometry, NMR, and amino acid analysis.

Thus, the bisubstrate analog inhibitor **34** was subjected to kinetic and structural studies with the IRK. Kinetic analysis of the bisubstrate analog inhibitor **34** showed that it was a rather potent inhibitor of IRK. Compound **34** behaved as a linear competitive inhibitor versus both ATP and peptide substrates. The K_i for **34** was 370 nM (extrapolated to 0 substrates) as an inhibitor of IRK, 190- and 760-fold lower than the K_m values of the substrates [$K_{m\ app}$ (ATP), 71 μM; $K_{m\ app}$ (IRS727) = 280 μM], respectively. In fact, the approximate binding energy for the interaction of **34** with IRK was similar to the binding energies of each of the individual peptide and nucleotide components. A detailed kinetic analysis showed that Compound **34** exhibited a slow off-rate from IRK ($t_{1/2} \sim 1$ min) (Parang et al., 2001).

The crystal structure of IRK in complex with **34** was determined at 2.7 Å resolution. The X-ray crystallographic structure confirmed that the nucleotide and peptide portions of **34** were binding to ATP and protein substrate-binding sites, respectively. As designed, this structure showed that the distance between the anilino nitrogen atom corresponding to the phenolic oxygen of tyrosine and γ-phosphorus was ~ 5.0 Å, consistent with the design plan and proposal that IRK favors a dissociative transition state (Ablooglu et al., 2000). Another important feature seen in the crystal structure is the presence of a hydrogen bond between the anilino nitrogen and Asp1132, similar to the phenolic hydroxyl group in tyrosine in the ternary complex structure. Proton removal from the hydroxyl of the Tyr residue occurs late in the tyrosine kinase reaction mechanism (Ablooglu et al., 2000, Kim & Cole, 1998), as described in Section 1.2, emphasizing the importance of this hydrogen bond. Thus, the Tyr residue phenol replacement with an anilino group, serving dually as a hydrogen bond donor and part of the linker, was probably an important part of the overall strategy. It was not clear a priori what the effect of the carbonyl oxygen in the linker would be. The X-ray structure showed that the carbonyl oxygen of the linker was coordinated to a Mg^{2+} in the active site (O-Mg distance was 2.2 Å), replacing a water molecule at that position in the ternary complex. Thus, the carbonyl oxygen actually may have contributed to the affinity of **34** for IRK.

It was proposed that the peptide moiety of **34** imparts both potency and specificity as an IRK inhibitor. Consistent with this prediction, the bisubstrate inhibitor was only a modest inhibitor against the PTK CT Src kinase (the preferred substrate peptide sequence for this kinase being quite different; Sondhi et al., 1998). Moreover, Compound **35**, which lacks the peptide moiety, had a K_i of 114 μM for IRK, was ~ 300-fold weaker as an inhibitor than Compound **34**, and was comparable to ATPγS alone (K_i of 210 μM). Therefore, the peptide specificity contributes significantly to the overall potency and selectivity of the inhibitor. This is an important feature, since this implies that it might be possible to generalize this approach to make a large set of kinase selective inhibitors.

On the other hand, the peptide moieties and charged phosphates will likely limit the bioavailability of these compounds and, therefore, their pharmacologic potential. To overcome this obstacle, peptidomimetics and alternative linkers can be used to substitute for these constituents in the next generation of bisubstrate analog inhibitors (Druker & Lydon, 2000).

3. Conclusions

This review highlights several strategies to design bisubstrate analog inhibitors for protein kinases, which may be selectively targeted towards particular protein kinases, based in part on their peptide substrate-specificity motif. Ligands that are able to disrupt signal transduction pathways by exploiting dual binding sites ultimately may be able to be used as potent and selective therapeutic agents. One recurrent theme is the importance of linker length and constitution for achieving maximal inhibition. This was found to be particularly important in exploiting the knowledge gained from previous analysis of the phosphoryl transfer transition state. While many of the compounds may have low bioavailability, there is considerable value in the development of such molecules, because they can provide tools for signal transduction and structural studies. In the future, it hopefully will be possible to use the knowledge gained from these synthetic and signalling studies to design useful therapeutics for human diseases.

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References

Ablooglu, A. J., Till, J. H., Kim, K., Parang, K., Cole, P. A., Hubbard, S. R., & Kohanski, R. A. (2000). Probing the catalytic mechanism of the insulin receptor kinase with a tetrafluorotyrosine-containing peptide substrate. *J Biol Chem 275*, 30394–30398.

Admiraal, S. J., & Herschlag, D. (2000). The substrate-assisted general base catalysis model for phosphate monoester hydrolysis: evaluation using reactivity comparisons. *J Am Chem Soc 122*, 2145–2148.

Aqvist, J., Kolmodin, K., Florian, J., & Warshel, A. (1999). Mechanistic alternatives in phosphate monoester hydrolysis: what conclusions can be drawn from available experimental data? *Chem Biol 6*, R71–R80.

Biscardi, J. S., Tice, D. A., & Parsons, S. J. (1999). c-Src, receptor tyrosine kinases, and human cancer. *Adv Cancer Res 76*, 61–119.

Bruice, T. C., & Benkovic, S. J. (2000). Chemical basis for enzyme catalysis. *Biochemistry 30*, 6267–6274.

Broom, A. D. (1989). Rational design of enzyme inhibitors: multisubstrate analog inhibitors. *J Med Chem 32*, 2–7.

Cole, P. A., Burn, P., Takacs, B., & Walsh, C. T. (1994). Evaluation of the catalytic mechanism of recombinant human Csk (C-terminal Src kinase) using nucleotide analogs and viscosity effects. *J Biol Chem 269*, 30880–30887.

Collins, K. D., & Stark, G. R. (1971). Aspartate transcarbamylase. Interaction with the transition state analogue N-(phosphonacetyl)-L-aspartate. *J Biol Chem 246*, 6599–6605.

Cowburn, D., Zheng, J., Xu, Q., & Barany, G. (1995). Enhanced affinities and specificities of consolidated ligands for the Src homology SH3 and SH2 domains of Abelson protein-tyrosine kinase. *J Biol Chem 270*, 26738–26741.

Cushman, M., Chinnasamy, P., Chakraborti, A. K., Jurayj, J., Geahlen, R. L., & Haugwitz, R. D. (1990). Synthesis of [beta-(4-pyridyl-1-oxide)-L-alanine4]-angiotensin I as a potential suicide substrate for protein-tyrosine kinases. *Int J Pept Protein Res 36*, 538–543.

Danenberg, P. V., & Danenberg, K. D. (1977). Inhibition of hexokinase by multisubstrate analogs. *Biochim Biophys Acta 480*, 351–356.

Druker, B. J., & Lydon, N. B. (2000). Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia. *J Clin Invest 105*, 3–7.

Frick, L., Yang, C., Marquez, V. E., & Wolfenden, R. (1989). Binding of pyrimidine-2-one ribonucleoside by cytidine deaminase as the transition-state analog 3,4-dihydrouridine and the contribution of the 4-hydroxyl groups to its binding affinity. *Biochemistry 28*, 9423–9430.

Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N., & Sawyers, C. L. (2001). Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science 293*, 876–880.

Ho, M., Bramson, H. N., Hansen, D. E., Knowles, J. R., & Kaiser, E. T. (1988). Stereochemical course of the phospho group transfer catalyzed by cAMP-dependent protein-kinase. *J Am Chem Soc 110*, 2680–2681.

Hubbard, S. R. (1997). Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. *EMBO J 16*, 5572–5581.

Hubbard, S. R., & Till, J. H. (2000). Protein tyrosine kinase structure and function. *Annu Rev Biochem 69*, 373–398.

Hunter, T. (1987). A thousand and one protein kinases. *Cell 50*, 823–829.

Hunter, T. (2000). Signaling—2000 and beyond. *Cell 100*, 113–127.

Kemp, B. E., Graves, D. J., Benjamini, E., & Krebs, E. G. (1977). Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J Biol Chem 252*, 4888–4894.

Kemp, B. E., Pearson, R. B., & House, C. M. (1991). Pseudosubstrate-based peptide inhibitors. *Methods Enzymol 201*, 287–304.

Kennelly, P. J., & Krebs, E. G. (1991). Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J Biol Chem 266*, 15555–15558.

Kim, K., & Cole, P. A. (1998). Kinetic analysis of a protein tyrosine kinase reaction transition state in the forward and reverse directions. *J Am Chem Soc 122*, 2145–2148.

Kruse, C. H., Holden, K. G., Pritchard, M. L., Field, J. A., Rieman, D. J., Greig, R. G., & Poste, G. (1988). Synthesis and evaluation of multisubstrate inhibitors of an oncogene-encoded tyrosine-specific protein kinase. *J Med Chem 31*, 1762–1767.

Lawrence, D. S., & Niu, J. (1998). Protein kinase inhibitors: the tyrosine-specific protein kinases. *Pharmacol Ther 77*, 81–114.

Lee, T. R., & Lawrence, D. S. (1999). Acquisition of high-affinity, SH2-targeted ligands via a spatially focused library. *J Med Chem 42*, 784–787.

Levitzki, A. (1999). Protein tyrosine kinase inhibitors as novel therapeutic agents. *Pharmacol Ther 77*, 81–114.

Lienhard, G. E., & Secemski, I. I. (1973). P^1,P^5-di(adenosine-5′)pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. *J Biol Chem 248*, 1121–1123.

Liu, M., Haddad, J., Azucena, E., Kotra, L. P., Kirzhner, M., & Mobashery, S. (2000). Tethered bisubstrate derivatives as probes for mechanism and as inhibitors of aminoglycoside 3′-phosphotransferase. *J Org Chem 65*, 7422–7431.

Loog, M., Uri, A., Raidaru, G., Järv, J., & Ek, P. (1999). Adenosine-5′-carboxylic acid peptidyl derivatives as inhibitors of protein kinases. *Bioorg Med Chem Lett 9*, 1447–1452.

Loog, M., Uri, A., Raidaru, G., Järv, J., & Ek, P. (2000). Bi-substrate analog ligands for affinity chromatography of protein kinases. *FEBS Lett 480*, 244–248.

Medzihradszky, D., Chen, S. L., Kenyon, G. L., & Gibson, B. (1994). Solid-phase synthesis of adenosine phosphopeptides as potential bisubstrate inhibitors of protein kinases. *J Am Chem Soc 116*, 9413–9419.

Mildvan, A. S. (1997). Mechanisms of signaling and related enzymes. *Proteins 29*, 401–416.

Musacchio, A., Saraste, M., & Wilmanns, M. (1994). High-resolution crystal structures of tyrosine kinase SH3 domains complexed with proline-rich peptides. *Nat Struct Biol 1*, 546–551.

Muszynska, G., Ekman, P., & Engström, L. (1993). Phospholipid-dependent and EGTA-inhibited protein kinase from maize seedlings. *Biochem Mol Biol Int 30*, 849–860.

Niu, J., & Lawrence, D. S. (1997). Nonphosphorylatable tyrosine surrogates. Implications for protein kinase inhibitor design. *J Biol Chem 272*, 1493–1499.

Page, M. I., & Jencks, W. P. (1971). Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. *Proc Natl Acad Sci USA 68*, 1678–1683.

Parang, K., Till, J. H., Ablooglu, A. J., Kohanski, R. A., Hubbard, S. R., & Cole, P. A. (2001). Mechanism-based design of a protein kinase inhibitor. *Nature Struct Biol 8*, 37–41.

Pearson, R. B., & Kemp, B. E. (1991). Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol 200*, 62–81.

Pehk, T., & Uri, A. (1997). Synthesis and structural characterization of conjugates of adenosine and tetra-aspartate, novel analogs of ATP. *Bioorg Med Chem Let 7*, 2159–2164.

Pinna, L. A., & Ruzzene, M. (1996). How do protein kinases recognize their substrates?. *Biochim Biophys Acta 1314*, 191–225.

Profit, A. A., Lee, T. R., & Lawrence, D. S. (1999). Bivalent inhibitors of protein tyrosine kinases. *J Am Chem Soc 121*, 280–283.

Profit, A. A., Lee, T. R., Niu, J., & Lawrence, D. S. (2001). Molecular rulers: an assessment of distance and spatial relationships of Src tyrosine kinase Sh2 and active site regions. *J Biol Chem 276*, 9446–9451.

Radzicka, A., & Wolfenden, R. (1995). Transition state and multisubstrate analog inhibitors. *Methods Enzymol 249*, 284–312.

Ricouart, A., Gesquiere, J. C., Tartar, A., & Sergheraert, C. (1991). Design of potent protein-kinase inhibitors using the bisubstrate approach. *J Med Chem 34*, 73–78.

Rossé, G., Séquin, U., Mett, H., Furet, P., Traxler, P., & Fretz, H. (1997). Synthesis of modified tripeptides and tetrapeptides as potential bisubstrate inhibitors of the epidermal growth factor receptor protein tyrosine kinase. *Helvetica Chimica Acta 80*, 653–670.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell 103*, 211–225.

Showalter, H. D., & Kraker, A. J. (1997). Small molecule inhibitors of the platelet-derived growth factor receptor, the fibroblast growth factor receptor, and Src family tyrosine kinases. *Pharmacol Ther 76*, 55–71.

Sondhi, D., Xu, W., Songyang, Z., Eck, M. J., & Cole, P. A. (1998). Peptide and protein phosphorylation by protein tyrosine kinase Csk: insights into specificity and mechanism. *Biochemistry 37*, 165–172.

Songyang, Z., Carraway, K. L., Eck, M. J., Harrison, S. C., Feldman, R. A., Saltiel, A. R., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B., Mayer, B. J., & Cantley, L. C. (1995). Catalytic specificity of protein-tyrosine kinases is critical for selective signaling. *Nature 373*, 536–539.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanatusa, H., Schaffhausen, B., & Cantley, L. C. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell 72*, 767–778.

Taylor, S. S., & Radzio-Andzelm, E. (1994). Three protein kinase structures define a common motif. *Structure 2*, 345–355.

Thiesing, J. T., Ohno-Jones, S., Kolibaba, K. S., & Druker, B. J. (2000). Efficacy of STI571, an abl tyrosine kinase inhibitor, in conjunction with other antileukemic agents against bcr-abl-positive cells. *Blood 96*, 3195–3199.

Toledo, L. M., Lydon, N. B., & Elbaum, D. (1999). The structure-based design of ATP-site directed protein kinase inhibitors. *Curr Med Chem 6*, 775–805.

Topaly, J., Zeller, W. J., & Fruehauf, S. (2001). Synergistic activity of the new ABL-specific tyrosine kinase inhibitor STI571 and chemotherapeutic drugs on BCR-ABL-positive chronic myelogenous leukemia cells. *Leukemia 15*, 342–347.

Traxler, P. (1998). Tyrosine kinase inhibitors in cancer treatment (part II). *Expert Opin Ther Patents 8*, 1599–1625.

Traxler, P. M., Wacker, O., Bach, H. L., Geissler, J. F., Kump, W., Meyer, T., Regenass, U., Roesel, J. L., & Lydon, N. (1991). Sulfonylbenzoylnitrostyrenes: potential bisubstrate type inhibitors of the EGF-receptor tyrosine protein kinase. *J Med Chem 34*, 2328–2337.

Uri, A., Järlebark, L., von Kügelgen, I., Schöberg, T., Undén, A., & Heibronn, E. (1994). A new class of compounds, peptide derivatives of adenosine 5′-carboxylic acid, includes inhibitors of ATP receptor-mediated responses. *Biorg Med Chem 2*, 1099–1105.

Vu, C. B. (2000). Recent advances in the design and synthesis of SH2 inhibitors of Src, Grb2 and ZAP-70. *Curr Med Chem 7*, 1081–1100.

Walsh, D. A., & Glass, D. B. (1991). Utilization of the inhibitor protein of adenosine cyclic monophosphate-dependent protein kinase, and peptides derived from it, as tools to study adenosine cyclic monophosphate-mediated cellular processes. *Methods Enzymol 201*, 304–316.

Xu, Q., Zheng, J., Xu, R., Barany, G., & Cowburn, D. (1999). Flexibility of interdomain contacts revealed by topological isomers of bivalent consolidated ligands to the dual Src homology domain SH(32) of abelson. *Biochemistry 38*, 3491–3497.

Xu, W., Doshi, A., Lei, M., Eck, M. J., & Harrison, S. C. (1999). Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol Cell 3*, 629–638.

Yarden, Y., & Ullrich, A. (1988). Growth factor receptor tyrosine kinases. *Annu Rev Biochem 57*, 443–478.

Yuan, C. J., Jakes, S., Elliot, S., & Graves, D. (1990). A rationale for the design of an inhibitor of tyrosyl kinase. *J Biol Chem 265*, 16205–16209.

Zhu, G., Decker, S. G., Maclean, D., McNamara, D. J., Singh, J., Sawyer, T. K., & Saltiel, A. R. (1994). Sequence specificity in the recognition of the epidermal growth factor receptor by the abl Src homology 2 domain. *Oncogene 9*, 1379–1385.