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## Peptides Targeting Protein Kinases: Strategies and Implications

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Protein kinases are important key regulators in most, if not all, biological processes and are linked with many human diseases. Protein kinases thus became attractive targets for drug design. Intracellularly active peptides that selectively interfere with kinase function and or kinase-mediated signaling pathways are potential drug compounds with therapeutic implications.

The protein kinases super family accounts for nearly 2% of the human genome and includes at least 500 kinases (49). Most of the biological processes regulated by signal transduction pathways involve protein phosphorylation catalyzed by protein kinases, including metabolism, transcription, cell cycle progression, and differentiation. They also play a critical role in intercellular communication during development and maintenance of eukaryotic organisms. Changes in protein phosphorylation states and kinase activity are associated with many human diseases. Thus they became important drug discovery targets. Indeed, it is estimated that more than 25% of drug development efforts in pharmaceutical and biotechnology companies are focused on the development of protein kinase inhibitors. Peptides can mimic protein-protein interactions and, thus, may provide a powerful means to regulate signaling events. This review highlights strategies to design such peptide inhibitors, focusing mainly on serine/threonine kinases, and discusses their advantages and limitations.

### Protein Kinase Structure is Highly Conserved

Protein kinases share extensive sequence and structural homologies, especially within their catalytic domain. Initially, this domain was classified into 11 conserved subdomains (31) and is folded architecturally into a two-lobe structure that contains a smaller NH<sub>2</sub>-terminal lobe, with a glycine rich loop (P-loop) for ATP binding, and a larger COOH-terminal domain, which contains a conserved activation loop (also called T-loop), which itself is regulated by phosphorylation (FIGURE 1A) (30, 69, 70). Substrate binding and its phosphorylation occur within the cleft formed between the two lobes (30, 69, 70). Even though protein kinases show such high similarity and perform similar phosphorylation catalysis, they display remarkable diversity in their substrate specificity. It appears that additional elements, such as distinct regions located outside the catalytic domain, separate regulator subunits, and adaptor molecules can affect the kinase activity and direct its cellular specificity toward substrates or signaling networks. Designing

optimal kinase-inhibitory compounds requires a comprehensive understanding of structure/specificity relationships and their implications in the cellular context. Clearly, high-resolution 3D structures of protein kinase, complexed with protein binding partners, is the best way to identify complementary interactions and to select the most specific one as a leading template for drug design. However, such structures are not always available, and other strategies are used, including bioinformatics, computational modeling, and high throughput screening (HTS). We can learn from the available structures that surface-surface contacts are major determinants controlling the function and specificity of protein kinases. Thus there is wide latitude for designing inhibitors that target these protein kinase interaction contacts. Peptides can mimic these interactions and, thus, are a good choice in this inhibitor-design approach. In the following sections, we classify peptide inhibitors by their mechanism of action (described in FIGURE 1B), and peptide sequences and their biological functions are listed in Table 1.

### Peptides Competing with Substrates

Protein kinases have preferences for substrates, which are determined by the so-called recognition motif. This sequence represents particular amino acids surrounding the phosphorylation site and was proven to be critical in substrate recognition by the protein kinase. Peptides that copy this motif have the potential to be substrate competitive inhibitors. The first discovered substrate competitive inhibitor was a natural 76-amino acid protein, PKI, which was initially copurified with the catalytic subunit of cAMP-dependent protein kinase and found to inhibit its activity (PKA) (75). PKI contains the typical PKA recognition motif RRNAL, in which the phospho-acceptor serine is replaced by alanine (underlined). Synthetic peptides derived from the corresponding "substrate-like" PKI sequence showed a high potency for inhibition (14, 28). The crystal structure of the complex PKA and a 20-residue peptide inhibitor verified interaction within the active site cleft and highlighted the role of the argi-

Table 1. Synthetic peptide inhibitors and their biological activities

Peptide Sequence (NH <sub>2</sub> ->>COOH)	Peptide Target (PK)	Derived From	Modification for Intracellular Activity	Biological Activity	Ref.
<i>Substrate</i>					
TYADFIASGRTGRRNAI <i>PKI-(6-22)-amide</i>	PKA	PS site of PKA		Affects hepatic gene expression Modulates neuronal synaptic activity	14, 28
KEAPPAPPQS(p)P <i>L803-mts</i>	GSK-3β	GSK-3 substrate recognition site of HSF-1	N-myristoylated	Anti-diabetic effect Anti-depressant-like effect Prevent Parkinson's Disease development	12a, 39a, 39b, 58
A: FARKGALRQ B: RFARKGALRQ KNV	PKCα/β	PS site of PKCα/β	N-myristoylated homeodomain antennapedia (Antp)	Reduce MARCK phosphorylation Inhibit neutrophil NADPH oxidase Affect neuronal cone morphogenesis	71, 73
SIYRRGARRWRKL	PKCζ	PS site of PKCζ	N-myristoylated	Blocks integrin dependent cell adhesion	45
LKKFNARRKLKGAILTMLA	CAMII kinase	PS site of CAMK II			10, 68
<i>Docking Sites</i>					
1. D-site MPKKKPTPIQLNPAPDG	ERK2	D-site of MEK1		Inhibits phosphorylation of ERK1/2	5
MQGKRKALKLNLFANPP	JNK1/2	D-site of MEK4		Inhibits phosphorylation of c-Jun and ATF2	35
<b>RPKRPTTLNLFPOVRSQDT</b> <i>(L)-JNK1 1 (SAPK Inhibitor I)</i>	JNK	JNK-binding domain (JIP-1)	HIV-TAT	Anti-diabetic effect in db/db mice Inhibits pancreatic β cells death Neuroprotection in injured brain	9, 28, 40
2. FXFP docking motif RRPRSPAKLS <b>FQFPS</b>	ERK	FQFP docking site Elk1		Mediated ERK-dependent insulin-induced oocytes maturation	22
3. HJ and αD regions GGYNQNHQKLFQL <i>KRX-014<sub>H151</sub></i>	PKB	HJ-αG region of PKB	N-myristoyl	Anti-apoptotic effect	46
GGRAGNQYL <i>KRX-702<sub>H105</sub></i>	PDK1	HJ-αG region of PDK1	N-myristoyl	Anti-apoptotic effect	46
4. Other protein docking sites HAKRRLIF <i>p21<sup>WAF1</sup></i>	CDK2	COOH-terminal cyclin-binding domain of P21 <sup>WAF1</sup>		In vitro inhibition of CDK2	82a
SQPETRTGDDDPHRLQLVLS- GNLIKEAVRRRLHSRRLO <i>FRATtide</i>	GSK-3	COOH-terminus of FRAT1	Expression vector	Blocks phosphorylation of Axin, β-catenin and tau	72
DIHVDPEKFAAELISRLEGVLRDR <i>GID</i>	GSK-3	GSK-3β-interacting domain of axin	Expression vector	Activates Wnt-dependent transcription Prevent nuclear export	26, 82b
<i>Cellular Targeting</i>					
EAVSLKPT <i>εV1-2</i>	PKCε	V1 domain of PKC-ε		Prevents cardioprotection in ischemic heart	38, 48
SLNPEWNET <i>βC2-4</i>	PKCβ	C2 domain of PKC-β		Inhibit PMA-induced PKC translocation in cardiac myocytes Delays insulin-induced oocyte maturation	60
DLIEEAASRIVDAVIEQVKAAGAY <i>S-Ht31</i>	PKA	AKAP	N-stearated	Affects synaptic transmission Inhibits oocyte maturation Changes in sperm motility	15, 53, 62, 74

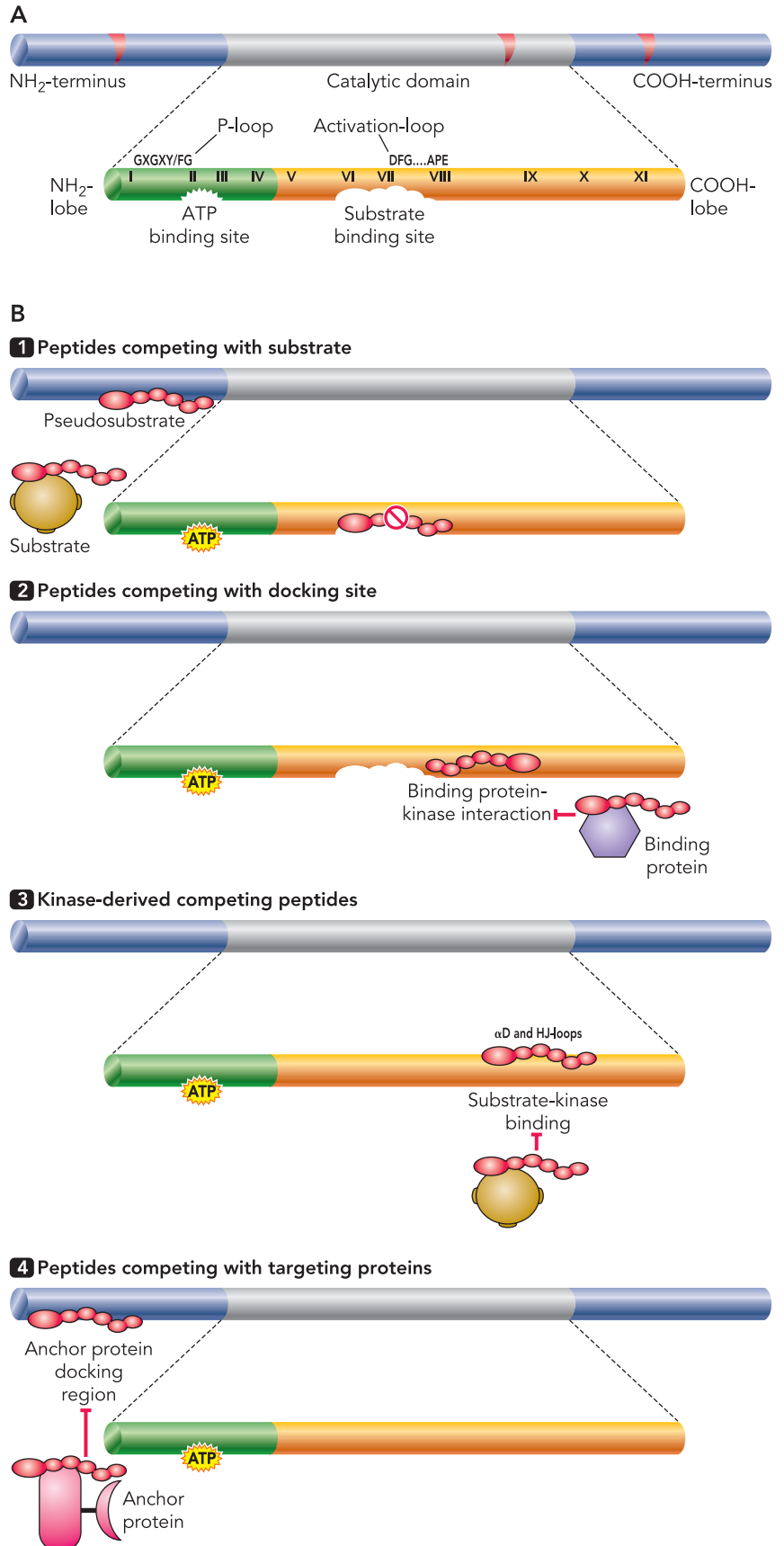
PS, psuedosubstrate. Recognition or docking motives are marked in bold.

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nine residues in the consensus motif as the primary determinants responsible for recognition (44, 52). Additional parts of the peptide distant from the RRNAL recognition motif, however, were shown to perform tight interaction with the kinase, for example, an arginine residue (numbered 15) located upstream of the recognition motif (44, 52). This emphasized the important contribution that surrounding residues may play in the inhibitory potency of the peptide as initially described by Glass et al. (28). Several peptides derived from the recognition motif of glycogen synthase kinase-3 (GSK-3) were proven to be potent and selective inhibitors as well. The peptides copied the GSK-3-recognition motif SXXXS(p), which is unique in that it required prephosphorylation of serine residue (25, 78). Serine phosphorylated peptides derived from GSK-3 substrates such as CREB or heat shock factor-1 (HSF-1), in which the phosphorylation site was replaced by alanine, were effective substrate competitive inhibitors (58). Furthermore, substitution of glutamic acid located upstream to SXXXS(p) motif improved the inhibitory potency of the peptide (58), suggesting that, likely to PKI peptide, the use of minimal recognition element sequence may not be sufficient for optimal inhibition, since the surrounding residues interact with the active site and influence efficacy.

Competitive inhibitors can be derived from an auto-inhibitory domain located outside the catalytic domain, the so-called pseudo-substrate domain (42, 67). The pseudo-substrate contains a copy of the kinase recognition motif, in which serine phosphoacceptor is replaced by nonphosphorylatable residue. The role of the pseudo-substrate region is to keep the kinase in an inactive conformation via its interaction with the substrate binding site (42, 67). Peptide inhibitors based on this "natural" mechanism may thus act as specific substrate-competitive inhibitors. The protein kinase C family, for example, consists of a pseudo-substrate sequence (about 13 amino acids) at the NH<sub>2</sub>-terminal region of each PKC isoform (43). Synthetic peptides derived from the pseudo-substrate sequence showed high selectivity toward inhibition of their corresponding PKC isoforms, such as  $\alpha$  and  $\beta$ ,  $\eta$ ,

$\theta$ , and  $\zeta$  (20, 43, 71). Biological activity of PKC pseudo-substrate peptides were reported in several cell systems, among them, inhibition of neutrophil NADPH



**FIGURE 1. Inhibitory mechanisms of peptides targeting protein kinases**

A: schematic presentation of domain structure of protein kinase with conserved bilobal catalytic domain composed of 11 conserved subdomains. Conserved domains including P-loop involved in ATP binding and activation loop with the DFG...APE motif are marked. Red triangles mark docking site position with proteins such as substrates, upstream regulators, and scaffold proteins. B: inhibition of protein kinase by peptides: 1) competition with substrate; 2) competition with docking sites derived from substrates; 3) competition with docking sites derived from the kinase; 4) competition with cellular targeting anchor proteins. Peptides marked in yellow indicate the position of derivation; peptides marked pink indicate the interaction with the target site; X describes disruption location; red chain represents the peptide inhibitor.

oxidase (73), neuron growth cone morphology (71), and integrin-dependent cell adhesion (45). Auto-inhibitory domains are found also in  $\text{Ca}^{2+}$ /CaM-dependent protein kinase II (CaMK-II), myosin light chain kinase (MLCK), and phosphorylase kinase (3, 10, 43). In CaM kinase II and MLCK, the regulatory domain contains a pseudo-substrate sequence that overlaps with the  $\text{Ca}^{2+}$ /CaM-binding motif and binds into the catalytic binding cleft in the absence of  $\text{Ca}^{2+}$ /CaM (68). Binding of  $\text{Ca}^{2+}$ /CaM neutralizes this inhibitory mechanism, leading to an “open” confor-

*“Protein kinases are usually a part of large complexes participating in signal transduction networks, and their activity/function may be controlled by protein-protein interactions.”*

mation of the catalytic domain (68). Synthetic peptides with this “double” motif derived from CAM kinase or MLCK were utilized as efficient inhibitors, and their mechanism of inhibition was described in detail (10, 68).

The peptide substrate competitive strategy is not always successful. This is due to limitations of peptides compared with substrates. Overall, substrates have more contact interactions with the kinase, which short peptides can miss, thus performing partial interaction with the kinase. In addition, substrate-kinase interactions are relatively weak to enable dissociation of the substrate from the enzyme. Indeed, substrate-competitive peptide inhibitors show relatively high  $K_i$  values at the micromolar range. However, one should take into consideration that in vitro potency does not always reflect the in vivo potential, mainly because in vitro assays use large excesses of substrates, whereas in cellular conditions the substrate's concentration is far below the micromolar range. Thus, in many cases, peptides show better efficacy in cells compared with cell-free conditions, and it is recommended to evaluate the peptide inhibitor in cellular conditions.

### Peptides Competing With Docking Sites

Protein kinases are usually a part of large complexes participating in signal transduction networks, and their activity/function may be controlled by protein-protein interactions. The docking interacting sites are distinct from the kinase active site, and these sites facilitate interaction with protein partners, such as substrates, upstream regulators, or adaptor proteins [see recent review (7)]. It is important to note that complex interactions can ensure specificity toward

signaling transmission. Disruption of these interactions may be a way to impair selective kinase-mediated signaling pathways, and, thus, peptide-based docking sites may be exploited for selective inhibition (see *scheme 1*). The mitogen-activated protein kinases (MAPK) family is a central player, coordinating diverse signaling pathways (12, 59, 65). The MAPK members (ERK, JNK, and p38) share very close structural similarity, but they provide a remarkably exquisite signal transmission (12, 59, 65). Apparently, specificity is achieved through specific docking interactions of each MAPK member with its cognate binding partners and results in assembly of three component units of the MAPK cascade, which includes the upstream activator MAPK kinase (MEK) and MAPK's downstream substrates (12, 59, 65). A high-affinity docking region, D-site [also termed KIM (for kinase interaction motif)], is found near the  $\text{NH}_2$ -termini of each MEK (i.e., MEK1, MEK2, MEK3, etc.), which enables a complex formation between MEKs and MAPKs (5, 21, 80). The D-site's consensus motif is typified by a cluster of basic residues followed by a hydrophobic submotif (5, 79). However, there is some variability in the amino acid composition, which likely determines specificity. Peptides derived from D-sites of MEKs may block their action specifically on their corresponding MAPKs. For example, the D-site peptide derived from MEK1 was used to inhibit ERK1/2 phosphorylation (5, 35). Peptides derived from the D-site of MEK4 prevented JNK1 and JNK2 activation and inhibited phosphorylation of their substrates c-jun and ATF2 (5, 35). In both cases, the peptides were selective and did not inhibit other MAPK members (5, 35). D-site-like motives were found also in other binding partners of MAPK, including its direct substrate S6 ribosomal protein kinase, p90 RSK, and MAP kinase dual specific phosphatases (MKPs) (63, 83). An interacting protein of JNK (JIP1) is a scaffold protein that facilitates the assembling of JNK-cascade components (19, 76, 77). Synthetic peptides corresponding to the D-site of JIP1 proteins were described in several works and showed high and selective inhibition potency toward JNKs in vitro and in vivo (6, 25, 36). JIP1 peptides had an important impact in several systems. The peptides controlled neuronal cell apoptosis enhancing neuroprotective effects (29), they blocked pancreatic  $\beta$ -cell death (9), and they provoked anti-diabetic activity in diabetic animal model (40). A different docking motif, FXFP, controls ERK recognition of its substrates. This site is an evolutionarily conserved docking site found in several transcription factor substrates of ERK, such as Elk-1, SAP-1, LIN-1, and Fos, and is shown to mediate high-affinity interactions between ERK and its substrate proteins (22, 27, 36). Utilizing synthetic peptides patterned after the FXFP motif was shown to antagonize MAPKs ability to phosphorylate its substrates and reduced MAPK-mediated signaling pathway (22, 37, 82).

A docking site within the catalytic domain of PDK-1 (3-phosphoinositide-dependent protein kinase-1) has been recently characterized. This site which is distinct from the ATP- or peptide substrate-binding sites interact with a "PDK1-interacting fragment," termed PIF, found in AGC kinases such as PKB, PKC, and PRK-2, which also serve as PDK-1 substrates (8). It has been suggested that the PIF motif enables substrate recruitment to PDK1, and, in addition, it may modulate PDK-1 catalytic activity (4). This was further demonstrated with the use of synthetic peptide derived from PIF (PIFtide) that enhanced PKB phosphorylation by PDK-1 (4). Inhibition of PDK-1 by PIFtide was not reported.

Although docking regions were derived mainly from cognate partners, a new technology presented a paradigm in which sequence-based peptide inhibitors are derived from the kinase itself. This technology, termed KinAce, offered a general strategy for generating inhibitors toward protein kinases. The method was based on the characterization of specific structural conserved regions termed  $\alpha$ D or HJ loop located within subdomains V or IX and X, respectively, and were identified as important determinants in substrate binding. The variability within the amino acid compositions suggested substrate recognition specificity (54). Thus it was assumed that peptide copies of these regions would compete with substrates. Peptides derived from PDK1 or PKB were shown to inhibit phosphorylation of their corresponding downstream targets (54). In addition, treatment with these peptides provoked cell growth inhibition of prostate cancer cells, correlating with the pro-survival activity of PDK1 and PKB (54). Other peptides derived from kinases such as these were implicated in diabetes and angiogenesis (2, 46).

Unlike the competitive inhibitors described previously, docking peptides prevent phosphorylation without grossly affecting the kinase activity. Therefore, inhibition of certain pathways may be separated functionally, namely, blocking phosphorylation of one substrate but not necessarily affecting the others. The docking peptides interact either with the kinase or the substrate, but, in both cases, the interaction substrate-kinase is blocked. The increasing number of reported peptide inhibitors based on docking interactions suggests that this approach is more successful compared with the substrate competitive-based approach. Although not fully understood, docking-docking interactions may be stronger compared with the temporal, weak interaction typically the nature of substrate-enzyme interactions.

### Peptides Competing with Cellular-Targeting Proteins

Many protein kinases are located at distinct cellular compartments and can be translocated from one compartment to the other. Subcellular localization is a

means for specific regulation of the kinase, since it brings the kinase into proximity with its localized substrates. Compartmentalization of protein kinases can be facilitated by interaction with adaptor protein also called anchor proteins. "Classical" examples are found in PKC and PKA. PKC isoforms are known to translocate from cytosol to particulate fractions in response to various stimuli. A family of adaptor molecules, termed RACKs (from receptors for activated C-kinase), was shown to interact with the various PKC isozymes and to facilitate their cellular translocation (61, 64). The RACK binding domain is a short sequence located in the PKC's NH<sub>2</sub>-terminal-subdomain and is specific for each isoform (60, 64). The translocation of a specific PKC isoform could be disrupted selectively using peptides derived from its RACK's docking region (60, 64). The use of such translocation peptide inhibitors had enabled the function of individual PKC isozymes to be determined. For example, peptides derived from the C2 region of PKC- $\beta$ II prevented hormone-induced membrane translocation of PKC- $\beta$  and inhibited insulin-induced *Xenopus* oocyte maturation (60). Peptides derived from V1 region of PKC- $\epsilon$  and PKC- $\delta$  had selectively blocked their cellular translocation in heart membranes, and their different cardio-protection effects were observed in ischemic hearts (38, 48).

The cellular regulation of PKA is controlled through interactions with a family of distinct, but functionally homologous, proteins: AKAPs (A-kinase anchor proteins) (16, 51). Each AKAP contains a typical amphipathic helix binding motif that anchors the PKA-regulatory subunit (R) into cellular sites of cAMP generation and PKA substrates (16, 51). Spatial regulation of PKA activity is dependent on gradient concentrations in c-AMP signaling and is critically important in development, differentiation, and apoptosis processes. Peptides encompassing the amphipathic helix binding motif of AKAP (also termed Ht31) were effective inhibitors for PKA recruitment to AKAP and served as useful tools for demonstrating the AKAP-/PKA-dependent signaling in various cell systems. For example, the use of AKAP peptide demonstrates the role of PKA in synaptic transmission (15, 62), in early oocyte maturation (53), and in sperm motility (74). Recently, a combination of bioinformatics and peptide array technologies were able to generate high-affinity binding peptides that were based on the AKAP binding motif. These peptides were selective toward some PKA-regulatory subunit isoforms and shown to be effective in displacing PKA from its cellular AKAP-anchoring sites (1, 11).

Additional example is found in *Wnt* signaling controlling GSK-3, and  $\beta$ -catenin *Wnt* pathway controls cell fate in embryo development and, in addition, was linked to cancer processes (56, 57). Complex formation facilitated by the scaffold protein Axin enables  $\beta$ -catenin phosphorylation by GSK-3 and enhances its proteosomal degradation (56, 57). *Wnt* inhibits

$\beta$ -catenin phosphorylation via activation of GBP/Frat protein, which disrupts Axin/GSK-3 interaction (6, 23). The crystal structures of GSK-3 complexed with Axin or Frat found that both proteins are bound to the same groove within GSK-3 (6, 18). Thus peptides corresponding to their interacting sequence with GSK-3 could compete with their binding to GSK-3 (6, 18). Treatment with a 39-residue peptide, FRATide, derived from FRAT, indeed prevented GSK-3 binding with Axin and the phosphorylation of both Axin and  $\beta$ -catenin (72); subsequently, expression of Axin-GID peptide derived from Axin prevented GSK-3 binding with FRAT, activated Wnt-dependent gene expression (81), and enhanced  $\beta$ -catenin phosphorylation and prevented FRAT-induced nuclear export of GSK-3 (26).

### Peptides in the Clinic

So far, we have described the potency of peptides as selective protein kinase inhibitors. However, a serious question arises concerning their bioavailability and potential in the clinic. Traditionally, peptides are considered less attractive when compared with “small molecule”-based drugs due to several limitations. Peptides do not cross cell membranes readily; they may be rapidly degraded in the tissue and or blood circulation; and, in addition, they cannot be administered orally. A great deal of progress, however, has been made in recent years to overcome part of these problems, and, indeed, many studies reported good in vivo efficacy of peptides (see Table 1). Permeability of peptides may be improved by conjugation with hydrophobic element. For example, attachment of fatty acids, such as myristic or palmitic acids significantly improve their cell permeability (17, 20, 41, 58). A different approach uses amino-acid sequences derived from proteins with membrane-translocation ability (also termed “PTD”), such as homeodomain transcription factors antennapedia (Antp), human immunodeficiency virus (HIV-1) transactivator TAT protein, and the signal sequence of Kaposi fibroblast growth factor (MTS) (24, 32, 34, 47). Stability of peptides can be improved by modification with various polymers that perverted protease activity. Alternatively, peptides may be incorporated into carriers, such as liposomes, which can deliver the peptide from circulation to target tissues (33, 50, 66). Finally, there are alternative routes developed for compounds that cannot be administered orally, such as buccal and nasal and dermal routes (33, 39, 55, 66). Recent work used phage display peptide libraries to identify selected peptide enhancers for transdermal delivery (13). Thus use of alternative approaches that can improve peptide bioavailability and delivery makes them potential candidates with therapeutic value.

### Conclusions

Selective inhibition of protein kinase is an extremely challenging goal in the area of drug discovery. This review highlighted the main strategies for the design of peptide inhibitors for protein kinases. The advantages of peptides as selective nontoxic compounds are well considered. Nevertheless, the limited bioavailability of peptides discouraged pharmaceutical companies from developing these compounds as drugs. However, with the rapid development in the field, it is worthwhile to reexamine the opportunities for peptides as potential drug compounds with appropriate technology and design.

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