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ADENOSINE-5'-CARBOXYLIC ACID PEPTIDYL DERIVATIVES AS INHIBITORS OF PROTEIN KINASES

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Abstract: A new class of protein kinase bisubstrate-analog inhibitors was designed on the basis of adenosine-5'-carboxylic acid derivatives, where a short peptide was attached to the 5'-carbon atom of the adenosine sugar moiety via a linker chain. The potency and selectivity of these inhibitors were adjusted by relevant combination of these structural fragments, resembling the structure of the bisubstrate complex of the peptide phosphorylation reaction.

Protein kinase inhibitors have attracted much attention as potential drugs, but also as valuable tools for investigation into the regulatory phosphorylation phenomena.[1,2] The strategies of design of these inhibitors have been based on the understanding of the phosphorylation reaction mechanism, involving the formation of ATP and peptide/protein bisubstrate complex. Firstly, protein kinases have been inhibited by compounds competing with ATP for its binding pocket on the enzyme molecule. These inhibitors include isoquinoline-sulfonamide derivatives,[3] staurosporine analogs,[4-6] and several other classes of compounds. Secondly, the inhibitors competing with the peptide or protein substrate for their binding site have been described.[7] This class of compounds was mostly comprised of peptides containing the substrate consensus motif. However, it has been shown for protein kinase A (PKA), that the peptides with the minimal substrate consensus motif were relatively poor inhibitors of the enzyme, and only longer peptides, containing the peptide sequence of the endogenous substrate analog inhibitors of PKA, had a more significant inhibitory effect.[8] Thirdly, as a combination of the approaches above, inhibitors directed simultaneously into the ATP and peptide binding sites have been designed. The pioneering work that followed this principle in the field of serine/threonine protein kinases was made by coupling of isoquinolinesulfonic acid with hexaarginine via a linker group.[9] Some of these compounds inhibited PKA and protein kinase C (PKC) at sub-micromolar concentrations. Further, an attempt was made to design a more accurate model of the bisubstrate complex by direct coupling of the serine residue of a specific PKA substrate Kemptide to adenosine nucleotides via a phosphodiester bond.[10] However, the latter compounds revealed only moderate inhibitory effects with sub-millimolar IC_{50} values. Thus it has been emerged that any efficient bisubstrate-analog inhibitor of a protein kinase should contain a suitable linker group which couples the ATP-competitive part of the inhibitor molecule to the peptide fragment. The present communication was focused on a more detailed analysis of the latter aspect of the design of protein kinase bisubstrate inhibitors and a novel class of these inhibitors was synthesised by coupling adenosine-5'-carboxylic acid (AdoC) with a peptide fragment via various linkers (Table 1).

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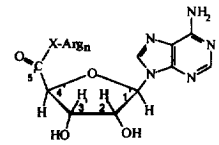
TABLE 1

The general formula of protein kinase inhibitors 

	Inhibitor[a)]	Linker X	n	MW[b]
I	AdoC(β-Ala)$_2$AlaArg$_6$	$NHCH_2CH_2C(O)NHCH_2CH_2C(O)NHCH(CH_3)C(O)$	6	1431.7
II	AdoC(Dpr)$_2$AlaArg$_6$	$NHCH_2CH(NH_3^+)C(O)NHCH_2CH(NH_3^+)C(O)NHCH(CH_3)C(O)$	6	1461.7
III	AdoC(β-Asp)$_2$AlaArg$_6$	$NHCH_2CH(COO^-)C(O)NHCH_2CH(COO^-)C(O)NHCH(CH_3)C(O)$	6	1519.7
IV	AdoCGlyArg$_6$	$NHCH_2C(O)$	6	1275.6
V	AdoC(β-Ala)Arg$_6$	$NH(CH_2)_2C(O)$	6	1289.6
VI	AdoC(GABA)Arg$_6$	$NH(CH_2)_3C(O)$	6	1303.6
VII	AdoC(Ahx)Arg$_6$	$NH(CH_2)_5C(O)$	6	1331.6
VIII	AdoC(Aoc)Arg$_6$	$NH(CH_2)_7C(O)$	6	1359.6
IX	AdoC(Aun)Arg$_6$	$NH(CH_2)_{10}C(O)$	6	1401.6
X	AdoC(Ahx)Arg$_4$	$NH(CH_2)_5C(O)$	4	1019.2
XI	AdoC(Ahx)Arg$_2$	$NH(CH_2)_5C(O)$	2	706.8
XII	Arg$_6$	-	-	955.2
XIII	Ac(Ahx)Arg$_6$	-	-	1110.1
XIV	Ado	-	-	267.3

[a)] The peptide and the linker were assembled by conventional Fmoc-peptide chemistry and 2'-, 3'-isopropylideneadenosine-5'-carboxylic acid was attached by coupling with BOP/Hobt activation.[11,12] The compounds were purified by an acetonitrile (0.1% TFA) gradient on a preparative C18 HPLC column. The products were analysed on a cation exchange column Mono S HR 5/5 (Pharmacia Biotech) and on a MALDI TOF mass-spectrometer Kratos Kompact IV. The experimental molecular mass values agreed well with the calculated values.

[b)] Calculated MW values.

Among the arginine-peptide containing compounds (Table 1) several potent inhibitors of PKA, PKC and Ca^{2+}-dependent protein kinase 1 (CDPK-1) [13] were found, while no significant inhibition of protein kinases CK1 and CK2 was observed (Table 2). This result was not surprising, as the substrate specificity of PKA and PKC [14] as well as of CDPK-1 [13] is clearly governed by positively charged amino acids, while protein kinases CK1 and CK2 phosphorylate only negatively charged peptide sequences.[15] Thus, the arginine-peptides were effective to direct the inhibitors towards PKA, PKC and CDPK-1. Similarly, it can be assumed that a negatively charged "anchoring" peptide fragments will direct the inhibitors into the active centre of protein kinases CK1 and CK2.

TABLE 2

Interaction of protein kinase A (PKA), protein kinase Cβ (PKC), Ca^{2+}-dependent protein kinase from maize seedlings (CDPK-1), protein kinase CK1 and protein kinase CK2 with adenosine-5'-carboxylic acid derivatives as protein kinase inhibitors.

Inhibitor	IC_{50} (μM) [e]				
	PKA [a]	PKC [b]	CDPK-1 [c]	CK1 [d]	CK2 [d]
I	4.3±0.3	2.8±0.3	16±4	>100	>30
II	2.6±0.3	1.2±0.1	31±3	5.6±0.4	17±3
III	11.1±0.6	27±2	>50	>30	>30
IV	4.0±0.2	10±1	67±7	>30	>30
V	1.8±0.1	3.0±0.2	38±7	>30	n. i. [f]
VI	1.3±0.2	1.5±0.1	19±2	>30	>30
VII	0.12±0.02	0.27±0.01	1.2±0.2	>30	>30
VIII	0.24±0.02	0.14±0.01	3.2±0.1	>30	>30
IX	0.33±0.02	0.32±0.02	4.9±0.5	>30	n. i. [f]
X	1.2±0.1	-	52±8	-	-
XI	13.8±1.0	6.9±0.3	35±3	-	-
XII	n. i. [f]	~100	>50	>100	n. i. [f]
XIII	>100	>100	>50	>100	n. i. [f]
XIV	141±11	278±8	116±18	126±15	>300

[a] The catalytic subunit of murine PKA was overexpressed and purified from *E.coli* strain BL21 (DE3).[16] The expression vector construct pRSETPKA was kindly provided by Dr. S.S. Taylor (La Jolla, California). PKA activity was assayed at 30°C in 60 mM Tris/HCl, pH 7.5, containing 3 mM 2-[N-morpholino]-ethanesulfonic acid, 0.002 % Triton X-100, 0.3 mM EDTA, 0.2 mg/mL bovine serum albumin, 100 μM of Kemptide (LRRASLG), 5 mM $MgCl_2$ and 30 μM [γ-^{32}P] ATP.

[b] Protein kinase C β isoform (PKC) was isolated from pig spleen.[17,18] PKC activity was assayed at 30°C in 50 mM Tris/HCl, pH 7.5, containing 0.002 % Triton X-100, 0.75 mM calcium acetate, 60 μg/mL phosphatidylserine, 1 μg/mL diolein, 0.3 mM EDTA, 0.2 mg/mL bovine serum albumin, 15 μM of substrate peptide KRAKRKTAKKR,[19] 5 mM $MgCl_2$ and 30 μM [γ-^{32}P] ATP.

[c] Ca^{2+}-dependent protein kinase (CDPK-1) was isolated from maize seedlings as described in.[13] The activity of CDPK-1 was assayed at 30°C in 45 mM Tris/HCl, pH 7.5, 0.005% Triton X-100, 0.7 mM $CaCl_2$, 0.4 mM EDTA, 0.4 mg/mL bovine serum albumin, 5 mM $MgCl_2$, 30 μM [γ-^{32}P]ATP and 0.2-0.4 μg of the protein kinase pool.

[d] Protein kinases CK1 and CK2 were purified from rat liver as described by Meggio et al.[20] The activity was assayed at 30°C in 60 mM Tris/HCl, pH 7.5, 1 mg/mL casein, 100 mM NaCl, 2.5% glycerol, 5 mM $MgCl_2$, CK1 or CK2 and 30 μM [γ-^{32}P] ATP.

[e] The reactions were monitored by transferring 25 μL aliquots onto 2 × 2 cm pieces of phosphocellulose paper, thereafter washed with 75 mM phosphoric acid five times and the bound radioactivity was measured. The IC_{50} values were obtained from the initial activity vs inhibitor concentration plots.

[f] n. i. - no inhibition at 100 μM concentration of the ligand.

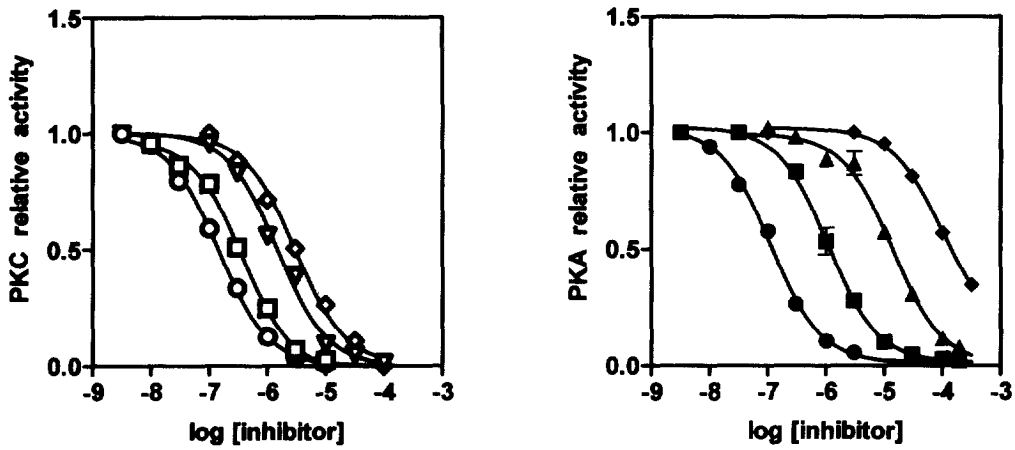


Figure 1. Inhibition of PKC (left) by inhibitors *VIII* (o), *IX* (□), *VI* (∇) and *V* (◊); inhibition of PKA (right) by inhibitors *IX* (●), *X* (■), *XI* (▲) and adenosine (◆).

The designed inhibitors contained structural elements of the both substrates of protein kinases, ATP and peptide. If tested separately, adenosine and peptide fragments alone had only a weak (adenosine) or even no inhibitory activity (Table 2). However, their combination *via* a linker group X increased the effectiveness of their interaction with the enzyme and yielded potent inhibitors of PKA, PKC and CDPK-1. The ionic status of X was varied using β-alanine, β-aspartic and 2,3-diaminopropionic acids for coupling the peptide and nucleoside parts, yielding differently charged linkers of the same backbone structure (compounds ***I***, ***II***, and ***III*** in Table 1). The IC_{50} values for these compounds with non-ionic and cationic linkers were similar, while the introduction of β-aspartic acid residues slightly decreased the potency of the inhibitors. This means that the ionic charges of the linker did not participate in the electrostatic stabilisation of the enzyme-inhibitor complex. As the linker part of the inhibitor presumably resides in the same region of the active site as the phosphate backbone of ATP in the reaction complex, these results were in agreement with the mutually close values of the affinities of ATP (K_d = 25 μM [21]) and adenine (K_d = 30 μM [22]) to PKA. Thus the charged phosphate moiety (as well as ribose) seems to make no clear contribution to the observed binding affinity. This could explain the failure of our attempts to increase the inhibitory potency of the bisubstrate-analog inhibitors by introducing charges into the linker region.

Secondly, the flexibility of the linker was varied by replacing the conformationally restricted peptide-like groups by aliphatic chains of various length (compounds ***IV*** to ***IX*** in Table 1). All these compounds were effective inhibitors of PKA, PKC and CDPK-1, while maximal inhibitory effect was clearly depending on the linker length (Fig.1a) and these dependencies were somewhat different for the investigated enzymes (Fig 2).

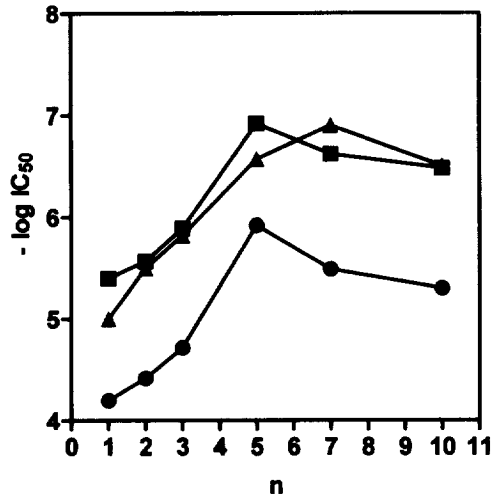


Figure 2. The effect of the linker group $NH(CH_2)_nC(O)$ length (n) on the effectiveness of inhibition of protein kinase A (■), protein kinase C (▲) and Ca^{++}-depending protein kinase from maize seedlings (●).

Further, the potency of the inhibitors was clearly dependent on the length of the peptide fragment attached (Fig.1b), pointing to a significant role of this part of the molecules in the specific interaction with the enzyme. Therefore it may be assumed that a variation of the peptide fragment structure, taking into account the details of the differential specificity of various protein kinases, may be a promising approach for a more selective targeting of these inhibitors.

The clear structural requirements for inhibition point to the importance of the compatibility of the inhibitor molecule with the binding sites for ATP and the peptide. These sites are located on different lobes of the catalytic subunit of PKA, where the upper lobe has the adenosine binding pocket and the lower lobe accommodates the peptide/protein binding site.[23] These lobes are believed to move towards each other and bring the substrates together during the catalytic act. On the other hand, the protein conformation should be rather opened to allow the substrate binding. Therefore the spatially separated location of these binding sites may well be the main structural factor determining the length and the chemical properties of the linker group.

Taken together, we have prepared and tested a series of a new type of effective protein kinase inhibitors, which are chemically stable and well soluble under physiological conditions and may be selectively targeted towards particular protein kinases proceeding from their peptide substrate specificity motif.

Acknowledgements

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