Bioorganic & Medicinal Chemistry Letters 19 (2009) 6098–6101

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Effect of the structure of adenosine mimic of bisubstrate-analog inhibitors on their activity towards basophilic protein kinases

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ARTICLE INFO

Article history:
Received 6 August 2009
Revised 4 September 2009
Accepted 5 September 2009
Available online 12 September 2009

Keywords:
Protein kinases
AGC kinases
PKA
PKB/Akt
ROCK
Bisubstrate inhibitors
Oligoarginine

ABSTRACT

Previously reported structural fragments that associate with the ATP-binding pocket of basophilic protein kinases were conjugated with D-arginine-containing peptides. Inhibitory potency of the resulting bisubstrate-analog inhibitors towards PKA and ROCK-II extended to subnanomolar range. The conjugates incorporating 2-pyrimidyl-5-amidothiophene fragment had the highest activity and at 100 nM concentration exhibited over 80% inhibition of most of the tested basophilic kinases of the AGC group.

In the last decade protein kinases (PKs) that regulate the activity of substrate proteins by catalyzing their phosphorylation have emerged as important drug targets.[1] Ten small-molecule inhibitors of PKs have been approved for clinical use in cancer treatment and more than 50 compounds are in clinical trials for drug development for cancer and other diseases.[2]

Due to the apparent ease of development of high-affinity low molecular weight inhibitors targeted to the well-defined hydrophobic adenine-binding cleft, most of the disclosed and studied inhibitors of PKs bind to the ATP-binding site. However, the design of ATP-competitive inhibitors has several downsides, including selectivity problems (in addition to 500 coded PKs in human genome, 1500 other proteins bind adenine nucleotides[3]) and the necessity to compete with high concentration of ATP in the cellular milieu.

The structure of protein substrate-binding domain of PKs is less conserved and several highly selective peptide inhibitors have been described.[4] However, longer peptidic structures are needed to achieve nanomolar potency which leads to problems with cellular transport and stability of the compounds.

The combined approach represented by the development of bisubstrate-analog (biligand) inhibitors that simultaneously associate with both ATP- and protein-binding domains of PKs could hence give selective and potent inhibitors of these two-substrate enzymes.[6,7]

Conjugates of adenosine analogs and arginine-rich peptides (ARCs)[5] designed by our research group inhibit several basophilic PKs from AGC group with nanomolar to subnanomolar potency.[6] The incorporation of arginine-rich transport peptides into the structure of ARCs provides them with cell-penetrative properties and allows their use for modulating intracellular protein phosphorylation.[8] The very high affinity of ARCs is retained upon their labeling with fluorescent dyes or immobilization that supports their application for biosensors.[9,10]

The crystal structure of a complex of the catalytic subunit of cAMP-dependent protein kinase (PKAc) with an ARC-type inhibitor Adc-Ahx-(D-Arg)$_2$-NH$_2$ (ARC-1034; Adc—adenosine-4′-dehydroxymethyl-4′-carboxylic acid, Ahx—6-aminohexanoic acid), the presumed lead scaffold of previously reported ARC-type inhibitors, was solved. According to the crystal structure, the nucleoside moiety of ARC-1034 was bound to the adenosine binding pocket of PKAc as expected and the C-terminal amide group of 6-aminohexanoic acid linker interacted strongly with the glycine-rich loop at the enzyme residues usually interacting with the β-phosphate group of ATP.[6]

This co-crystal structure served as a basis for the development of ARC-type inhibitors of the third generation, incorporating 2 flexible linkers and a chiral spacer (Fig. 1). Ahx was used as the first (flanking Adc) linker whose structure was optimized according to the results previous structure–activity studies.[5a] The chiral spacer, a D-amino acid residue, directs the peptide chain towards the

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doi:10.1016/j.bmcl.2009.09.026

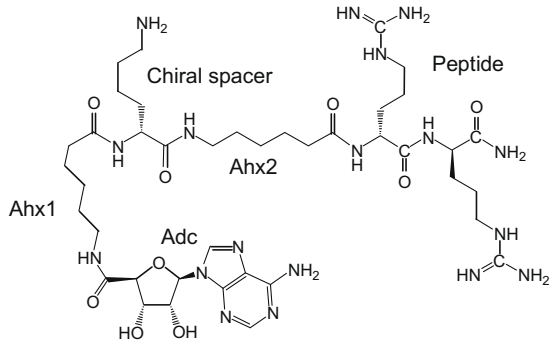
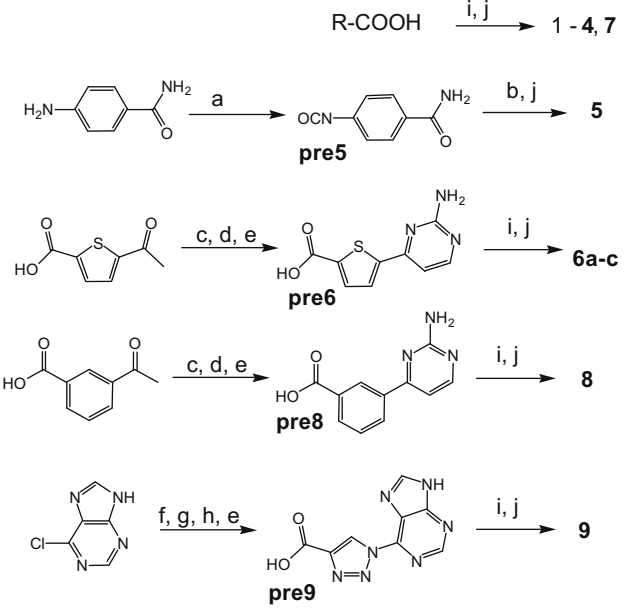


Figure 1. Adc-Ahx-(D-Lys)-Ahx-(D-Arg)$_2$-NH$_2$, compound **1** (ARC-1012), was used as the template for the design of the new bisubstrate inhibitors.

binding site for arginines of a PK substrate. The second linker (Ahx2 in Fig. 1) is the tether between the chiral spacer and the oligoarginine fragment. The latter moiety associates with substrate proteins/peptide binding site of basophilic PKs.[6] Compound **1** (ARC-1012, compound **XV** in Ref. 6; Fig. 1) inhibits several basophilic protein kinases at nanomolar level (IC_{50}-values 50–800 nM at 100 μM ATP concentration).

Here, we report on bisubstrate-analog inhibitors where Adc moiety of compound **1** was replaced with fragments that according to the literature and reported co-crystal structures bind to the kinase in a similar way as adenosine. The structures of both the optimized linker and the peptide were primarily left unchanged. From this series of compounds the conjugate with the highest inhibitory potency was chosen for the extension of the oligoarginine moiety from (D-Arg)$_2$-NH$_2$ to (D-Arg)$_6$-NH$_2$ to increase the potency of the inhibitors to the subnanomolar region.[6]

Bisubstrate inhibitors and their precursors were prepared according to the Scheme 1. Protected peptides were synthesized on the Rink-amide resin according to the conventional Fmoc peptide synthesis methodology. Adenosine mimic that contained a free carboxylic acid group was coupled to the peptide N-terminus on solid phase with HBTU/HOBt activation. Differently, N-terminal acetylation of the peptide was performed with acetic anhydride. Unstable precursor **pre5** was synthesized via treatment of 4-aminobenzamide with phosgene and it was used without separation in the reaction with the resin-bound peptide to prepare the conjugate **5**. Synthesis of **pre6** followed mainly the previously described procedure.[11] 5-Acetylthiophene-2-carboxylic acid was treated with *N*,*N*-dimethylformamide dimethyl acetal followed by the reaction with guanidine in the presence of a base (K_2CO_3 or CH_3O-Na) to form the aminopyrimidine ring. Hydrolysis of the ester gave triethyl ammonium salt of the carboxylic acid (**pre6**) that was used in the coupling reaction with the peptide on resin. A similar procedure was used for synthesis of **pre8** (starting from 3-acetylbenzoic acid). For the synthesis of **pre9**, 6-chloropurine was reacted with hydrazine, followed by reaction with sodium nitrite in acetic acid to give 6-azidopurine. Ethyl propiolate, ascorbic acid and $CuSO_4$ (0.1 equiv in water) were directly added to the resulting mixture. The overall yield of this one-pot procedure (including three separate steps) was only 7%. The low yield may be caused by instability of 6-azidopurine intermediate that exists in different tautomers.[12] Hydrolysis of the ester gave the carboxylic acid **pre9** that was thereafter coupled with the peptide. Other precursors were purchased from commercial sources. Protection groups were removed and the target conjugates were cleaved from the Rink-amide resin by treatment with mixture of TFA/H_2O/triisopropyl silane 90/5/5.



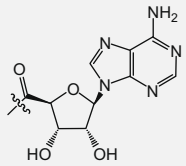
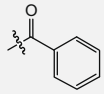
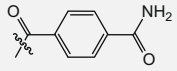
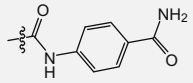
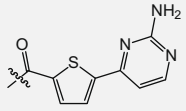
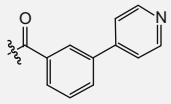
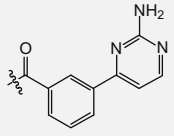
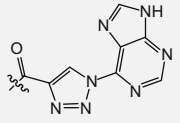
Scheme 1. Synthesis of adenosine mimic precursors and their conjugates with arginine-rich peptides. Reagents and conditions: (a) triphosgene, DCE, 0 °C, 10 min; (b) H_2N-peptide–resin; (c) *N*,*N*-dimethylformamide dimethyl acetal, toluene, reflux; (d) guanidine, K_2CO_3, DMF, reflux; (e) TEA, H_2O; (f) hydrazine; (g) $NaNO_2$, AcOH; (h) ethyl propiolate, ascorbic acid, $CuSO_4$; (i) H_2N-peptide–resin, HBTU/HOBt; (j) TFA/H_2O/triisopropyl silane (90/5/5).

The activity of the synthesized compounds was determined towards three PKs (PKAcα[13], PKBγ[6,13] and ROCK-II[9], Table 1). For comparison, the data for the previously characterized compound **1**[6] are also listed in the Table 1. Compound **2** incorporating an acetyl group caused almost no inhibition of PKAc- and PKBγ-catalyzed reactions while weak binding to ROCK-II could be established for this compound. Benzoyl derivative **3** was clearly a better inhibitor than **2** and insertion of 4-amido group (compound **4**) led to a 10-fold increase of potency that points to the formation of hydrogen bond with the hinge region of the kinase. The application of the urethane tether in **5** instead of amide group in **4** led to the 10-fold decrease in inhibitory potency. However the compound **4** is still a 30–500-fold weaker inhibitor than its adenosine counterpart **1**.

The heterocyclic moieties used in the novel conjugates were chosen according to the overlay of X-ray structures of the co-crystals of complexes of PKAc with 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid-based inhibitors,[11] from one side, and Adc-containing ARC-type bisubstrate inhibitors, from the other side.[6] As both groups of inhibitors have a carboxyl groups positioned in the same region of PKAc, the heterocyclic adenosine-mimicking fragments could be efficiently conjugated with peptides in similar way as it was performed in case of adenosine mimics in ARCs, taking advantage of the optimized linker in the latter conjugates. Very high potency of the conjugates **6a**, **7** and **8** towards tested AGC kinases points to the adequacy of this hypothesis. Compound **6a** was a stronger inhibitor than its adenosine counterpart **1** towards all three tested kinases. Compound **7** revealed some ROCK-II selectivity that is in accordance with the selectivity of the adenosine-mimicking fragment.[14] Compound **8** with similar structure was a weaker inhibitor of PKBγ and ROCK-II leading to some PKAc selectivity for this compound. Triazole-purine derivative **9** was a much weaker inhibitor than expected although being structurally isosteric to the potent inhibitor **6a**. The decreased activity of compound **9** may be caused by tautomeric equilibria leading to re-positioning of NH hydrogen bond donor group to N-7 of the purine that eliminates its potential for a hydrogen bond with the kinases.

Based on the highest potency of the **pre6**-derived inhibitor in the series of compounds containing the short (D-Arg)$_2$-NH$_2$ peptide (**6a** in Table 1) conjugates that comprised peptides containing 6

Table 1
Inhibitory potency and affinity of the conjugates incorporating various adenosine mimics and the (D-Arg)$_2$-NH$_2$ peptide

Compds	R	Inhibition of PKAc, α IC_{50}[a,b] (nM)	Inhibition of PKBγ IC_{50}[a,b] (nM)	Displacement of ROCK-II K_d[a] (nM)
R-Ahx-(D-Lys)-Ahx-(D-Arg)$_2$-NH$_2$				
1		54 ± 6[6]	774 ± 13[6]	<3
2		na 3 mM	>2 mM	123,000 (3.91 ± 0.19)
3		183,000 (3.74 ± 0.18)	~360000	8000 (5.10 ± 0.15)
4		23,000 (4.64 ± 0.14)	28000 (4.55 ± 0.18)	177 (6.75 ± 0.18)
5		240,000 (3.62 ± 0.25)	~900000	6100 (5.21 ± 0.28)
6a		14 (7.85 ± 0.18)	270 (6.57 ± 0.14)	<3
7		390 (6.41 ± 0.12)	900 (6.05 ± 0.31)	3.8 (8.42 ± 0.14)
8		61 (7.21 ± 0.16)	4600 (5.34 ± 0.17)	36 (7.44 ± 0.24)
9		3250 (5.49 ± 0.17)	27,000 (4.57 ± 0.21)	800 (6.10 ± 0.25)

[a] The values of pIC_{50} or pK_d with 95% confidence intervals (na = not active) are listed in the brackets.
[b] 100 μM ATP was used in kinetic assays, K_m-values were 20 μM for PKA and 100 μM for PKBγ.

D-arginine residues were prepared. Table 2 lists the activity data for these compounds. These compounds displaced at low concentration

Table 2
Inhibition potency of conjugates of 5-(2-aminopyrimidin-4-yl)thiophene-2-carboxylic acid

Compds	Inhibition of PKAc,α IC_{50}[a,b] (nM)	Inhibition of PKBγ IC_{50}[a] (nM)
6a Pre6-Ahx(D-Lys)Ahx(D-Arg)$_2$-NH$_2$	82 (7.08 ± 0.15)	270 (6.57 ± 0.14)
6b Pre6-Ahx(D-Lys)Ahx(D-Arg)$_6$-NH$_2$	5.5 (8.26 ± 0.27)	12 (7.92 ± 0.30)
6c Pre6-Ahx(D-Ala)Ahx(D-Arg)$_6$-(D-Lys)-NH$_2$	4.9 (8.31 ± 0.23)	42 (7.38 ± 0.19)

[a] Values in the brackets express pIC_{50}-s or pK_d-s with 95% confidence intervals.
[b] 1000 μM ATP was used in kinetic assay of PKA to minimize the distortions caused by the tight binding conditions.

the fluorescent probe ARC-583 (K_D = 3.6 nM for ROCK-II)[9] from the complex with ROCK-II, but as the probe does not afford the determination of exact K_d-values for compounds with dissociation constants K_d <3 nM[9] the data are not listed in Table 2.Compound **6b** was about 10–25-fold more potent than **6a** towards PKAc and PKBγ, which correlates well with previous data for their adenosine counterparts. Compounds **6b** and **6c** have similar inhibitory potency towards PKA but **6b** is 3.5-fold more active than **6c** towards PKBγ. Previously it was shown that the D-alanine spacer between the linkers increased the selectivity of ARCs towards PKAc.[6]

Selectivity of **6b** towards 50 PKs was tested in a panel of PKs (Invitrogen, SelectScreen Biochemical Kinase Profiling, Z'-LYTE Assay; Table 3) at 100 nM concentration of the inhibitor. It was assumed that the basophilic kinases would constitute the 'target group' of the inhibitors and the 'negative controls' would be represented by the acidophilic kinase CK1, kinases of the CMGC group (CDK2, GSK isoforms), and the tyrosine kinase Src. The same

Table 3
Inhibition efficiency of **6b** (100 nM) towards protein kinases

Protein kinase	Kinase group	% of inhibition
AMPK A1/B1/G1	CAMK	41 (2)[a]
Aurora A	Other	46 (4)
CaMK-Iδ	CAMK	25 (8)
CaMK-IIα	CAMK	10 (1)
CaMK-IIβ	CAMK	22 (6)
CDK2/cyclin A	CMGC	6 (5)
CHK1	CAMK	82 (0)
CHK2	CAMK	33 (5)
CK1α1	CK1	−22 (2)
DAPK3 (ZIPK)	CAMK	44 (3)
GSK3α	CMGC	8 (1)
GSK3β	CMGC	4 (4)
MELK	CAMK	83 (3)
MSK1	AGC	112 (9)
MSK2	AGC	112 (7)
p70S6K	AGC	99 (1)
PAK3	STE	69 (4)
PAK4	STE	21 (4)
PAK6	STE	70 (0)
PDK1	AGC	65 (9)
PIM1	CAMK	97 (2)
PIM2	CAMK	95 (1)
PKA Cα	AGC	104 (2)
PKBα (Akt1)	AGC	97 (4)
PKBβ (Akt2)	AGC	80 (5)
PKBγ (Akt3)	AGC	100 (2)
PKC α	AGC	92 (3)
PKC βI	AGC	100 (0)
PKC βII	AGC	104 (3)
PKC δ	AGC	97 (4)
PKC ε	AGC	103 (5)
PKC γ	AGC	108 (1)
PKC η	AGC	106 (5)
PKC ι	AGC	24 (8)
PKC μ	AGC	93 (6)
PKC θ	AGC	91 (1)
PKCζ	AGC	38 (11)
PKD2	CAMK	79 (0)
PKD3	CAMK	90 (9)
PKG1	AGC	105 (0)
PKG2	AGC	78 (3)
PRKX	AGC	106 (0)
ROCK-1	AGC	106 (2)
ROCK-II	AGC	106 (2)
RSK1	AGC	97 (0)
RSK2	AGC	103 (1)
RSK3	AGC	97 (1)
SGK1	AGC	97 (2)
SGK2	AGC	91 (1)
SRC	TK	26 (3)

[a] Percent of inhibition of the kinases (**6b**, $C = 100$ nM; ATP, $C \approx K_m$), as relative to that in control incubations where the inhibitor was omitted (means of duplicate determinations). Values in parentheses are differences of results of duplicate measurements.

kinases were previously used for characterization of the adenosine counterpart (ARC-1028, compound **XXII** in Ref. 6) of **6b**.[6]

Generally, **6b** inhibited strongly the same basophilic kinases as its adenosine counterpart ARC-1028. PKBα, PKBγ, PKG1, PKG2, SGK-s, PKD-s, PIM-2 and PKCμ were more potently inhibited by **6b** than ARC-1028, while the adenosine counterpart was more active with CHK-s and PAK-s.[6]

In conclusion, several aromatic structures were used as adenosine mimics to produce bisubstrate inhibitors for basophilic PKs with up to subnanomolar potency. Novel compounds comprising synthetic non-natural fragments and D-arginine residues are expected to be resistant to enzymatic degradation and thus have potential for application in experiments with live cells and tissues.

Acknowledgments

The research was supported by grants from the Estonian Science Foundation (6710) and the Estonian Ministry of Education and Sciences (SF0180121s08).

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