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ADENINE-ARGININE MIMETICS AS BISUBSTRATE ANALOG INHIBITORS OF cAMP-DEPENDENT PROTEIN KINASE

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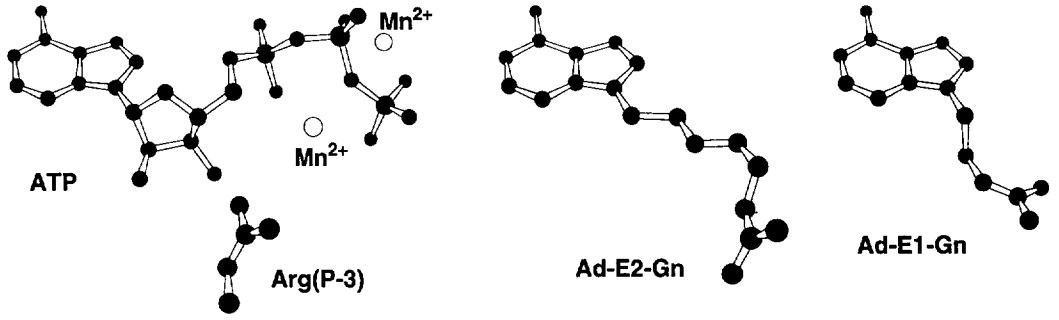
Abstract: Simple bisubstrate analogs, Ad-E1-Gn, Ad-E2-Gn, and Ad-E3-Gn, are designed to be proto-type adenine-arginine mimetic structures. Both Ad-E1-Gn and Ad-E2-Gn inhibit PKA with IC_{50} values similar to that of adenosine at 100 μM ATP and are more potent inhibitors than adenosine at physiologically relevant 2 mM ATP. Ad-E3-Gn is 10-fold less potent than the other two analogs. Copyright © 1996 Elsevier Science Ltd

Rapid growth in the number of known, identified protein kinases calls for the development of specific inhibitors, both as tools for pharmacological studies and as potential leads toward drug design targeting signal transduction pathways. We have been interested in combining the binding determinants of both the phospho group donor, ATP, and the phospho group acceptor, the peptide substrate, into a multisubstrate analog. Inhibitors with such structural characteristics are most likely to discriminate the subtle variation at the active site of different protein kinases with high selectivity. Bisubstrate analogs linking a known ATP-antagonist, 5-isoquinoline sulfonamide, and poly arginine peptides have been reported to show discrimination between PKA and PKC.[1] However, these bisubstrates contained as many as seven amino acid residues and some of the less complicated analogs were not as potent as the simple 5-isoquinoline sulfonamide inhibitors (the H-series).[2]

Figure 1. Design of adenine-arginine mimetics.

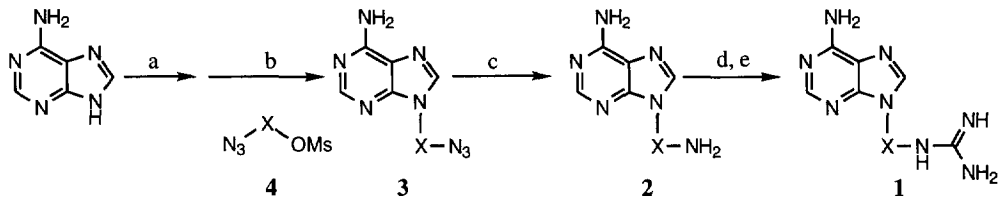


The availability of a ternary PKA-ATP-peptide complex[3] has made it possible to rationally design multisubstrate inhibitors for PKA. We recognized that the guanidino group of the *P-3* arginine is placed near the ribose unit of ATP, within distance for forming hydrogen bonds. The distance between the N(9) of adenine and the N(δ) of arginine is 8.2 Å. Compounds, containing a guanidino group tethered to an adenine ring and satisfying the above spatial requirement, may be inhibitors of PKA. In our modeling study, we found that an ethyl ether unit could be a suitable linker (Figure 1). The 3D-structural homology has been shown for several protein kinases.[4] If the strategy of adenine-arginine mimetics can yield inhibitors for PKA, it could be adopted

for designing inhibitors of other kinases, especially those having substrate specificities very different from PKA. To test this idea, we synthesized three proto-type designs for adenine-arginine mimetics, Ad-E1-Gn (**1a**), Ad-E2-Gn (**1b**), and Ad-E3-Gn (**1c**), and studied their ability to inhibit PKA.

The preparation of the guanidino adenines is summarized in Scheme 1. Derivatization of adenine at the 9-position was carried out in dry DMF under N_2 by alkylating the adenine anion with azido mesylate (**4a**, **4b**, or **4c**).[5] The azido adenine product (**3a**, **3b**, or **3c**) was separated from unreacted adenine by silica gel flash chromatography using a solvent system containing EtOAc and MeOH in 50–70% yields. Catalytic hydrogenolysis of the azido adenine gave the corresponding amino adenine (**2a**, **2b**, or **2c**) in quantitative yields. Conversion of the amino adenine into the corresponding guanidino adenine (**1a**, **1b**, or **1c**) was carried out at rt in a 1:1 mixture of MeOH and water (0.5 mmol/mL) containing 2.5 equiv of NaOH and 1.5 equiv of *S*-methyl thiopseudourea sulfate.[6] All three guanidino adenines were purified by HPLC. 360 MHz ^{1}H NMR spectra for the azido, amino, and guanidino derivatives were consistent with the desired products. 90 MHz ^{13}C NMR spectra for the three guanidino compounds were also obtained.[7]

Scheme 1. Synthesis of guanidino adenines, Ad-E1-Gn, Ad-E2-Gn, and Ad-E3-Gn.



4a X=CH_2CH_2	**3a** (53%, mp 174–5 °C)	**2a** (98%, mp 202–3 °C)	**1a (Ad-E1-Gn)**[g]
4b X=$CH_2CH_2OCH_2CH_2$	**3b** (70%, mp 75–6 °C)	**2b** (94%, mp 127–8 °C)	**1b (Ad-E2-Gn)**[g]
4c X=$CH_2CH_2(OCH_2CH_2)_2$	**3c** (66%, mp 96–7 °C)	**2c** (N.D.,[f] mp 87–8 °C)	**1c (Ad-E3-Gn)**[g]

[a]NaH, DMF, 0 °C to rt. [b]mesylate 4a, 4b, or 4c, rt, 16–24 h. [c]Pd, H_2. [d]1.5 equiv NaOH, 1.5 equiv *S*-methyl-thiopseudourea sulfate, 24 h. [e]HPLC, Vydac C-18 column, 5 or 10% AcCN, 0.1% TFA. [f]Unable to completely form dry solid. Yields not determined. [g]Ref. 6.

The inhibitory potency of adenosine and the three guanidino adenines were tested against the holoenzyme of PKA in the presence of cAMP.[8] The amount of radioactivity incorporated into Kemptide[9] from [γ-^{32}P]ATP was measured by a modified method of Glass et al..[8,10] At 100 μM ATP, Ad-E1-Gn and Ad-E2-Gn showed IC_{50} values in the 10^{-4} M range, comparable to that of adenosine (Figure 2). The IC_{50} value for Ad-E3-Gn was 10 times higher than adenosine. The K_d value for ATP was reported to be 10 μM.[11] Using our IC_{50} = 160 μM for adenosine, we estimated the K_i against ATP binding to be 15 μM.[12,13,14] This is consistent with the reported K_d value of 30 μM for adenosine.[11] By the same method, the K_i values for Ad-E1-Gn and Ad-E2-Gn (Table 1) were estimated to be 3 to 4 times the K_d value of ATP. One of the critical issues in designing protein kinase inhibitors is the need to overcome the high intracellular levels of ATP (2 to 10 mM). A true ATP antagonist, such as adenosine, will have unrealistically high IC_{50} values at physiological ATP concentrations. We have

determined the IC_{50} for adenosine to be 1.1 mM at 2 mM ATP in the current study, and an IC_{50} = 2.4 mM for adenosine under similar conditions was previously determined using a spectrophotometric method.[15] At high ATP concentrations (2 mM), Ad-E1-Gn and Ad-E2-Gn bind the enzyme better than adenosine, with IC_{50} values less than half of the ATP concentration, whereas the less potent inhibitor, Ad-E3-Gn, has an IC_{50} value 3 times higher than the ATP concentration.

Figure 2. Inhibition curves[12] of PKA by adenosine and guanidino adenines at 100 μM ATP.

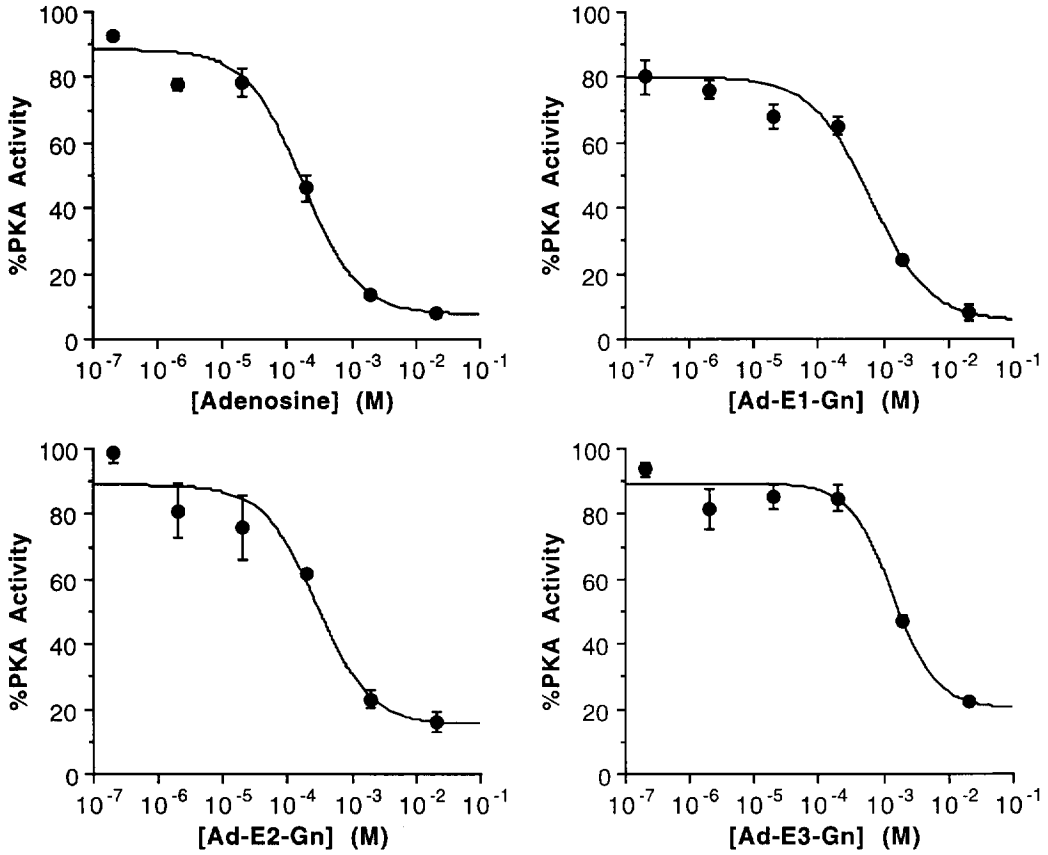


Table 1. Inhibitory properties of guanidino adenines on PKA.

Inhibitors	IC_{50} (μM)[12]			Est. K_i (μM)[13,14]
	at 100 μM ATP	at 2 mM ATP	ratio	
Adenosine	160	1100	6.9	15
Ad-E1-Gn	420	790	1.9	38
Ad-E2-Gn	320	760	2.4	29
Ad-E3-Gn	1700	6200	3.6	150

As summarized in Table 1, the ratio of the IC_{50} values at high vs. low ATP concentrations is smaller for Ad-E1-Gn and Ad-E2-Gn than for adenosine. This suggests that a guanidino group properly tethered to the adenine ring effectively behaves as a bisubstrate analog. The less potent Ad-E3-Gn supports the hypothesis that the distance between the guanidino group and the adenine group is critical for recognizing the bisubstrate analog. The binding specificity of the guanidino adenines is not solely based on the presence of an adenine moiety. In the presence of 2 mM ATP, a non-charged 9-substituted adenine, Ad-E3-OTHP,[16] was not able to block 50% of PKA activity at concentrations as high as 10 mM, supporting the requirement of a positively charged group in addition to the adenine moiety.

In summary, we have found simple analogs for adenosine, which exhibit inhibitory properties for PKA similar to that of adenosine. More significantly, the guanidino adenines (**1a**, **1b**, and **1c**) and the amino adenines (**2a**, **2b**, and **2c**) do not contain the ribose ring structure, hydroxyl groups or chiral centers of adenosine or ATP. The chemistry involved in the synthesis of these compounds can readily be modified to include substituents. The compounds reported here are useful intermediates for building other specific inhibitors of protein kinases, as well as inhibitors of other ATP-utilizing enzymes.

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5 Azido mesylates **4a**, **4b**, and **4c** were prepared from the corresponding chloro alcohols via azide substitution in water at 80 °C and subsequent conversion into mesylate with mesyl chloride. The intermediate azido alcohol for **4a** was volatile, and the organic layer extract of the azide substitution reaction was used directly for mesylation. Complete conversion of chloride into azide was confirmed by ^{1}H NMR.
6 The conversion, monitored by HPLC (Vydac C-18 column), was found to be 80% after 24 h, with no significant improvement after longer reaction time or addition of more reagent.
7. 90 MHz ^{13}C NMR: **1a** (42.4, 45.1, 119.7, 146.2, 146.5, 150.6, 151.6, 158.8 ppm); **1b** (41.3, 44.2, 68.5, 69.0, 118.1, 144.9, 145.8, 148.9, 150.8, 157.4 ppm); **1c** (41.4, 44.3, 68.4, 69.0, 69.8, 117.7, 144.5, 145.2, 148.8, 149.9, 157.6 ppm). For **1c**, the chemical shift at 69.8 ppm was assigned as two overlapping signals.
8. The reaction mixture contained 10 mM $MgCl_2$, 0.002% Triton X-100, 2 μM cAMP, 100 μM Kemptide, 2 mM, or 100 μM ATP, 20 mM Tris at pH 7.4, and 2 μg/mL PKA holoenzyme (Sigma). The experiment was carried out in triplicate at 30 °C for 5 min, 25 μL of reaction mixture was spotted on Whatman P81 paper marked with one inch square grids. The paper was washed with 75 mM H_3PO_4 (3x), then methanol, and allowed to dry. The amount of radioactivity was quantified on a Molecular Dynamics PhosphoImager.
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12. The inhibition curve was calculated by fitting the data points to $y = (a-d) / (1+ (x/c)^b) + d$ with weight $1/y^2$. The constants a, b, c, and d were calculated using Sigma Plot. The IC_{50} values were determined from the inhibition curve.
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14. The K_i values are estimated from the equation: $K_i = IC_{50} / (1 + [S] / K_m)$. The IC_{50} values are those at 100 μM ATP. K_m = 10 μM for ATP was used in the calculation.
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16. 9-(2-(2-(2-(2-Tetrahydropyranyloxy)ethoxy)ethoxy)ethyl)adenine was prepared via alkylation of the adenine anion.

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