Nucleoside Inhibitors of Rhodopsin Kinase†

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ABSTRACT: The specificity of the ATP-binding site of rhodopsin kinase was studied with adenosine analogues that are competitive inhibitors. Systematic changes in the ribose ring (position 5′) and the purine ring (positions 2, 6, 7, 8, and 9) and determination of the inhibitory properties of these analogues lead to the following conclusions: (1) The N^6 nitrogen in the purine ring is essential for binding at the active site, which may explain the marked preference for ATP rather than GTP as substrate. (2) The configuration of the sugar moiety is critical for the binding. (3) Positions 2, 3, and 8 of the purine ring, as well as the polyphosphate chain, play a minor role in substrate recognition by rhodopsin kinase. (4) ATPγS is a good substrate for rhodopsin kinase (thus rhodopsin phosphorothioate, a phosphatase-resistant product, can be formed in order to study the role of phosphorylation in rod outer segments). Pyrrolopyrimidine derivatives are very potent inhibitors of rhodopsin kinase. The K_i of one of these, sangivamycin, is 180 nM. Sangivamycin in solution assumes the anti conformation, as determined by nuclear Overhauser measurement. These measurements show that the most potent inhibitors of rhodopsin kinase, sangivamycin and toyocamycin, occur in solution preferentially in the anti conformation. Many nucleotides and nucleosides tested that are not inhibitors are syn, and many that are inhibitors form a mixture of syn and anti. The hypothesis that inhibitors may have a conformation intermediate between syn and anti was strengthened by testing a cyclic nucleoside locked in an anti conformation. This compound, an 8,3′-cycloadenosine derivative, is a good inhibitor of rhodopsin kinase with K_i = 10 μM. This suggests that rhodopsin kinase binds nucleosides in an intermediate syn/anti conformation. The low K_i value for sangivamycin allows us to block phosphorylation in whole rod outer segments with submicromolar concentrations, lower than those required for blocking other protein kinases.

The photoreceptor protein rhodopsin is the major protein of rod cell outer segments and becomes phosphorylated upon exposure to light (Bownds et al., 1972; Kühn & Dreyer, 1972; Frank et al., 1973). This phosphorylation process is proposed to be one of the mechanisms of quenching of photolyzed rhodopsin (Sitaramayya & Liebman, 1983). Rhodopsin's phosphorylation occurs on its cytoplasmic surface where as many as eight to nine phosphates are introduced by a very specific kinase, rhodopsin kinase [reviewed by Kühn (1984) and Hargrave et al. (1988)]. Rhodopsin is a member of the class of receptors that are coupled to G-proteins, and phosphorylation as a mechanism of receptor desensitization appears to be a shared property of this receptor class. Another well-studied example of such a mechanism is that of the β-adrenergic receptor (Mayor et al., 1987; Benovic et al., 1987a).

The enzyme that catalyzes rhodopsin phosphorylation, rhodopsin kinase, acts independently of second messengers (such as cAMP or cGMP). The phosphorylation velocity and extent appear to be regulated mostly, if not exclusively, by the concentration of ATP and the amount of photolyzed rhodopsin (Kühn, 1978; Palczewski et al., 1988a).

The interaction between rhodopsin and rhodopsin kinase requires multipoint attachment of the kinase to its substrate (Palczewski et al., 1989). Since phosphorylation occurs only after rhodopsin undergoes light-induced conformational rearrangement, the kinase is sensitive to structural changes of the photolyzed substrate (Kühn, 1978; Palczewski et al., 1989).

Rhodopsin kinase uses MgATP much more effectively than MgGTP as a phosphate donor (Shichi & Somers, 1978) and requires extra Mg^{2+} for maximum activity (Palczewski et al., 1988a). The enzyme is competitively inhibited by adenosine (Shichi & Somers, 1978) and by some adenosine analogues (Palczewski et al., 1988b). All of the analogues previously tested that have changes in the purine moiety have been ineffective as inhibitors of rhodopsin kinase. It also appears that the ribose moiety of the ATP is very sensitive to changes (Palczewski et al., 1988b).

Understanding of the specificity of an enzyme leads to better design of inhibitors and activators and to elucidation of in vivo regulation of the enzyme, and it serves as the basis for improved methods of purification based on affinity chromatography, etc. For several protein kinases the specificity of the protein and nucleotide-binding site is well-known (Glass & Krebs, 1979; Turner et al., 1985; Thomas et al., 1987). Extensive studies have been performed to elucidate the nucleotide-binding sites of calmodulin-dependent protein kinase (Kwiatkowski & King, 1987), phosphorylase kinase (Cheng et al., 1988), and cAMP- and cGMP-dependent protein kinases (Flockhart et al., 1984). Although the importance of a particular region for interaction between nucleotides and kinases has been studied, much less is known about the active conformation of the nucleotide.

The present study had three major objectives: (a) to determine the specificity of rhodopsin kinase's nucleoside-binding

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site with respect to the structure and conformation of its substrate and inhibitors, (b) to search for a potent inhibitor of rhodopsin kinase to facilitate studies of its physiological function, and (c) to determine the characteristics of nucleotide ligands to be considered as candidates for affinity chromatography of rhodopsin kinase.

Materials and Methods

The following chemicals were obtained from Sigma Chemical Co.: ATP disodium salt, adenine, 8-bromoadenosine, 8-bromo-AMP, 2-chloroadenosine, 5′-(*N*-ethylcarbamoyl)-adenosine, 6-chloropurine riboside, N^6-cyclopentyladenosine, 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole, D-ribose 5-phosphate, tubercidin 5′-monophosphate, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane (BTP),[1] and other chemicals not listed below.

N^6-(4-Aminobenzyl)adenosine, adenosine 5′-*O*-(2-thiodiphosphate) trilithium salt, adenosine 5′-*O*-thiomonophosphate dilithium salt, adenosine 5′-*O*-(1-thiotriphosphate) *S*-isomer tetralithium salt, and adenosine 5′-*O*-(3-thiotriphosphate) tetralithium salt were purchased from Boehringer Mannheim Biochemicals.

Sangivamycin, sangivamycin amidoxime, sangivamycin amidine, and toyocamycin were obtained from the National Institutes of Health, National Cancer Institute (Natural Products Branch), Division of Cancer Treatment. Toyocamycin (50 mg) was desalted by chromatography on silica gel (1.6 × 10 cm column of 15–40-μM beads). The column was developed with the organic fraction of 2-butanol–acetic acid–water (5:3:2). The nucleoside fractions were collected and lyophilized. Naphthalenesulfonamide and isoquinolinesulfonamide derivatives (M1-9, H 7, H 8, and HA 1004) were kindly provided by Dr. Hiroyoshi Hidaka from Nagoya University School of Medicine.

8,2′-Anhydro-8-mercapto-9-(β-D-arabinofuranosyl)adenine, 8,2′-anydro-8-oxy-9-(β-D-arabinofuranosyl)adenine, and 8,3′-anhydro-8-oxy-9-(β-D-xylofuranosyl)adenine were the generous gift of Dr. Janet L. Rideout from Burroughs Wellcome Co. (Research Triangle Park, NC). 8,5′-Anhydro-8-oxy-9-(β-D-ribofuranosyl)adenine was kindly provided by Dr. Ray A. Olsson (University of South Florida, Tampa, FL). [γ-^{32}P]ATP and [γ-^{35}S]ATP were purchased from New England Nuclear, Boston, MA.

Synthesis of N^6 Analogues. N^6-(Aminooctyl)adenosine, N^6-(aminoethyl)adenosine, and N^6-octyladenosine were prepared by the same method (Fleysher et al., 1968). The 6-chloropurine ribose (3 mmol) was refluxed for 4 h with a 10-fold excess of the appropriate amine or diamine and a 50-fold excess of triethylamine in absolute ethanol (70 mL). N^6 analogues were crystallized twice from ethanol.

The structure and purity of the compounds was confirmed by NMR, elemental analysis, and HPLC (C18 column, Phenomenex; Partisil 5S0S3, 150 × 4.6 mm; flow rate, 1 mL/min; buffer A, 0.1% H_3PO_4 in H_2O; buffer B, 0.1% H_3PO_4 in CH_3CN; A → B in 30 min). Retention times on the column and λ_{max} values (determined in 20 mM BTP buffer, pH 7.5) were as follows: N^6-(aminooctyl)adenosine, 48.8 min, 268 nm; N^6-octyladenosine, 68.1 min, 268 nm; N^6-(aminoethyl)adenosine, 44.0 min, 266 nm. Anal. Calcd for N^6-(aminooctyl)adenosine: C, 54.8; N, 21.3; H, 7.70. Found: C, 54.3; N, 20.8; H, 7.58. Anal. Calcd for N^6-(aminoethyl)adenosine hydrate: C, 42.7; N, 24.9; H, 6.27. Found: C, 43.1; N, 23.6; H, 6.26.

Concentrations of Reagents. The concentration of rhodopsin was determined at 498 nm by assuming a molar extinction coefficient of 40 600 (Wald & Brown, 1953) and a molecular weight for rhodopsin of 40 000 (Hargrave et al., 1983). The concentrations of nucleotides and nucleosides (the most potent inhibitors of rhodopsin kinase) were measured spectrophotometrically by assuming the following molar absorption coefficients: ATP and its phosphorothioate analogues, 15 400 at 259 nm (Bock et al., 1956); adenosine, 17 000 at 260 nm; adenine, 13 500 at 260 nm; inosine, 13 500 at 249 nm (Weast, 1979); sangivamycin, 12 400 at 278 nm (at neutral pH); 2-chloroadenosine, 12 200 at 263 nm; tubercidin 5′-monophosphate, 6700 at 278 nm; toyocamycin 5′-monophosphate, 13 600 at 275 nm; formycin 5′-monophosphate, 9300 at 303 nm (Hughes et al., 1983). The concentrations of sangivamycin amidoxime and sangivamycin amidine were determined by using the absorption coefficient for sangivamycin. The concentrations of cyclic nucleosides 8,5′-anhydro-8-oxy-9-(β-D-ribofuranosyl)adenine, 8,2′-anhydro-8-mercapto-9-(β-D-arabinofuranosyl)adenine, 8,2′-anhydro-8-oxy-9-(β-D-arabinofuranosyl)adenine, and 8,3′-anhydro-8-oxy-9-(β-D-xylofuranosyl)adenine were determined by assuming a molar absorption coefficient of 13 900 at 268 nm (pH 1) as determined for 2′,3′-*O*-isopropylidene-8,5′-cycloadenosine (Harper & Hampton, 1972).

In the present work we determined the molar absorption coefficients for N^6-(aminooctyl)adenosine, N^6-octyladenosine, and N^6-(aminoethyl)adenosine to be 18 000, 18 200, and 18 500 at 268 nm.

Preparation of Rhodopsin Kinase. Rhodopsin kinase was purified from bovine retinas as described by Palczewski et al. (1988b), except that for Tris buffer we substituted 20 mM BTP buffer, pH 7.5. The specific activity was 500–850 nmol of P_i transferred min^{-1} (mg of kinase)$^{-1}$.

Assay for Rhodopsin Kinase Using Urea-Washed ROS. Rhodopsin kinase activity was determined as described by Palczewski et al. (1988a). The reaction mixture (300 μL) contained 10 μM urea-washed ROS, 1 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (8 × 10^4 cpm/nmol), 10–60 μL of rhodopsin kinase solution, and 2 mM DTT in 20 mM BTP buffer, pH 7.5. The reaction was terminated by addition of 10% TCA, and the excess radioactive ATP was removed (Palczewski et al., 1988a).

For determination of the K_m values for ATP and ATPγS, the ATP or ATPγS concentration was varied from 0.3 to 16 μM and from 3 to 160 μM, respectively, at a rhodopsin concentration of 10 μM. Double-reciprocal plots of initial velocity versus substrate concentration were linear, as expected for a typical competitive inhibitor. Inhibition constants were obtained by linear regression analysis of Dixon plots and averaging of results from at least two separate experiments (for the most potent inhibitors, at least four). Concentrations of inhibitors were in the range of 0.2–10 times K_i. For weak inhibitors (K_i > 100 μM), K_i values were estimated from I_{50} determinations at ATP concentrations of 25, 50, and 100 μM

[1] Abbreviations: BTP, 1,3-bis[[tris(hydroxylmethyl)methyl]amino]-propane; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; $f_i(j)$, the NOE enhancement expressed as the percent enhancement of peak *i* observed upon saturation of peak *j*; NECA, 1-(6-amino-9*H*-purin-9-yl)-1-deoxy-*N*-ethyl-β-D-ribofuranuronamide [5′-(*N*-ethylcarbamoyl)adenosine]; NOE, nuclear Overhauser enhancement; ROS, rod cell outer segments; sangivamycin, 4-amino-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine-5-carboxamide; sangivamycin amidine, 4-amino-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine-5-carboxamidine; sangivamycin amidoxime, 4-amino-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]-pyrimidine-5-carboxamidoxime; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TCA, trichloroacetic acid; toyocamycin, 4-amino-5-cyano-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine.

Table I: Kinetic Parameters for ATP Components and Their Phosphorothioate Analogues[a]

no.	compound	K_i (μM)	K_m (μM)	ref
1	ATP		1.6	*b*
2	ATPγS		27	
3	ATPαS (*S*-isomer)	35		
4	ADP	12		*b*
5	ADPβS	22		
6	AMP	5.4		*b*
7	AMPS	22		
8	adenosine	4.0		*b*
9	adenine	8.7		
10	ribose 5-phosphate	no effect		
11	$P_iP_iP_i$	14		
12	P_iP_i	24		

[a] The inhibition constants for inhibitors of rhodopsin kinase were determined as described under Material and Methods. The determinations were performed at 30 °C with 10 μM bovine rhodopsin (as urea-washed rod cell membranes) as a substrate. [b] From Palczewski et al. (1988b).

by using the approach described by Cheng and Prusoff (1973).

NMR Measurements. NMR experiments were performed on a Varian VXR-300 instrument at 25 °C. All nucleosides and nucleotides were dissolved in D_2O buffer and the pD was adjusted to 7.0 with DCl and NaOD. Sangivamycin precipitated above pD 3.2; therefore, all experiments were run with this nucleoside at pD 3.2. All solutions were deoxygenated by bubbling of argon through the sample for 20 min.

NMR assignments were made by using 2D COSY and T_1 relaxation time measurements. Nuclear Overhauser enhancements (NOE) were determined by calculating the percent change in the peak area upon saturation of other selected peaks in the spectrum. NOE's were defined as $f_i(j)$ = [(area of peak i when j is saturated) − (area of i)]/(area of i). Peak areas were determined by using the integration routines supplied with the Varian VXR software. The decoupler power was adjusted so that only enough power was used to accomplish saturation, and the decoupler power was held constant in each set of experiments on a given sample. Each NOE is the average of at least three experiments.

Molecular models were constructed and energy-minimized by using MicroChem XP 2.5 (Chemlab, Inc.) on a Macintosh II. Ball and Stick II 2.2 (Norber Muller, Lunz, Austria) was used for molecular graphics on a Macintosh II computer.

Protein Determinations. Protein concentrations were measured by using the micro-Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

SDS-PAGE. SDS-PAGE was performed (Laemmli, 1970) with 12% acrylamide gels in a Hoeffer minigel apparatus.

Results and Discussion

Effect of ATP Components on Rhodopsin Kinase. Kinetic parameters K_m and K_i for ATP and its components have been determined for rhodopsin kinase (Table I). It is interesting that even the adenine (**9**) base of ATP is a relatively good competitive inhibitor of rhodopsin kinase (K_i = 8.7 μM). Addition of ribose (to give adenosine, **8**) or the mono-, di-, or triphosphoribose moiety (forming **6**, **4**, and **1**, respectively) does not greatly lower the K_i (or K_m). On the other hand, it has been shown that modification of the ribose component does make a nucleotide a less effective inhibitor (Palczewski et al., 1988b); only those ribose-containing analogues that have preserved the C2′-OH residue and the ribose configuration are accepted by the active site of rhodopsin kinase. However, the phosphoribose part of the nucleotide itself does not act as an inhibitor of rhodopsin kinase, even at a concentration of 40 mM (**10**; Table I). Therefore, the portion of ATP that appears to contribute energetically to the interaction with rhodopsin kinase is the adenine moiety. Polyanionic phosphates (**11**, **12**) inhibit the kinase activity, probably due to interaction with the phosphate-binding site of ATP.

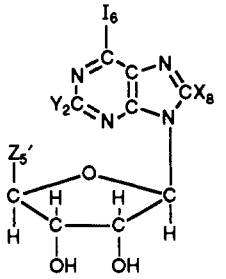
ATPγS Is a Substrate for Rhodopsin Kinase. Nucleoside phosphorothioates have been widely used in various biochemical studies. Enzyme mechanism and regulation [reviewed by Eckstein (1985)], preparation of protein phosphatase-resistant substrate (Li et al., 1988; Cassel et al., 1983), affinity purification of phosphorothioate derivatives of proteins (Sun et al., 1980), and interaction of nucleoside 3′,5′-cyclophosphorothioates with proteins and application in molecular biology [reviewed by Eckstein and Gish (1989)] are only selected examples of the applications of phosphorothioate analogues of nucleotides. ATPγS has also been used to study phosphorothiolation of the rim protein from ROS (Szuts, 1985).

Our goal was to test whether ATPγS is a substrate for rhodopsin kinase. If so, then it could be used to prevent the action of protein phosphatase(s) on phosphorylated rhodopsin in whole ROS. Indeed, ATPγS (**2**) is a relatively good substrate for rhodopsin kinase (Table I) with K_m = 27 μM and a V_{max} similar to that for ATP (data not shown). It is interesting that even such small changes of the nucleotide structure as replacement of oxygen by sulfur in the phosphate moiety change not only the K_m substantially for ATP and its thio analogue (**2**, **3**) but also the K_i values for ADP and AMP (**4** and **6**) and their phosphorothioate derivatives (**5**, **7**). The other important observation was that the overall extent of phosphorothiolation of rhodopsin was low. Under conditions in which rhodopsin kinase incorporated 5–6 mol of phosphate from ATP per mole of rhodopsin, only 2 mol of phosphorothioate was incorporated.[2]

Effect of Modification of Adenine Positions 2 and 8 on Inhibition of Rhodopsin Kinase. The selectivity for binding of nucleosides (nucleotides) may be due in part to a conformational effect on the sugar–base torsion angle (N-glycosylation bond). In order to help predict which conformer (syn or anti) of a nucleoside (nucleotide) binds to rhodopsin kinase, X_8- (**13**, **14**), Y_2- (**15**), and Z_5- (**1**, **16**, **17**) substituted analogues were tested (Table II). 8-Bromo-AMP (**13**) is a good example of a nucleotide in the syn conformation due to the electrostatic repulsion between the 8-Br group and 5′-P_i (Sarma et al., 1974). This nucleotide does not inhibit rhodopsin kinase. When restrictions on this conformation are relaxed (e.g., bromoadenosine, **14**), the analogue becomes a good inhibitor of the kinase. For analogues with substituents in position 2 (**15**), which should exist in the anti conformation, tight binding is observed (Table II). This also suggests that the 2-position of the purine ring is not involved in recognition of or in the preference of ATP over GTP as a substrate (Shichi & Somers, 1978; Palczewski et al., 1988b). NECA (**16**) is also a good inhibitor of rhodopsin kinase. In the solid state, this analogue exists in a syn conformation (the glycoside torsion angle is similar to that for 8-bromoadenosine, **14**) (Tavale & Sobell, 1970) with a novel H-bond between N-3 and $N^{5'}H$ (Moos et al., 1989). However, the conformation in solution has been determined to be intermediate between the syn and

[2] In a control experiment, we used protein kinase A and peptides LeuArgArgAla*Ser*LeuGly and LeuArgArgAla*Thr*LeuGly with ATP or ATPγS to determine whether the limited extent of phosphorylation of rhodopsin and rhodopsin kinase could be due to differences in the rate of phosphorylation of Ser and Thr residues. Indeed, a serine residue is at least 10 times more readily phosphorothiolated than a threonine residue by protein kinase A, probably due to its more nucleophilic character (data not shown).

Table II: Kinetic Parameters for 2-, 6-, 8-, and 5′-Substituted Analogues of Purine Nucleotide or Nucleoside[a]



no.	X_8	Y_2	$Z_{5'}$	I_6	K_i (μM)	K_m (μM)	ref
1	H	H	$CH_2(P_i)_3$	NH_2		1.6	*a*
8	H	H	CH_2OH	NH_2	4.0		
13	Br	H	CH_2P_i	NH_2	no effect		
14	Br	H	CH_2OH	NH_2	20		
15	H	Cl	CH_2OH	NH_2	29		
16	H	H	$C(=O)NHCH_2CH_3$	NH_2	16		
17	H	H	CH_2SO_3H	NH_2	80		*a*
18	H	H	CH_2OH	Cl	no effect		
19	H	H	CH_2OH	OH	no effect		*a*
20	H	H	CH_2OH	$NH(CH_2)_2NH_2$	no effect		
21	H	H	CH_2OH	$NH(CH_2)_8NH_2$	slightly stimulated		
22	H	H	CH_2OH	$NH(CH_2)_7CH_3$	no effect		
23	H	H	CH_2OH	NHC_6H_4-4-NH_2	activation (max 1.7 times)		
24	H	H	CH_2OH	NH-c-C_5H_9	500		
25	H	H	CH_2OH		no effect		*a*

[a] From Palczewski et al. (1988b).

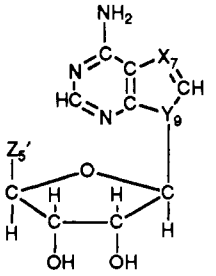
anti conformation (Stolarski et al., 1980; Akhren et al., 1979). Thus, it appears that the more potent inhibitors of rhodopsin kinase may be those that exist in the anti or in an intermediate conformation.

The 5′ position is also involved in the binding to rhodopsin kinase. Although deletion of phosphate (to give the nucleoside **8**) has no deleterious effect on interaction of the analogue with the kinase, the substitution of sulfonate for phosphate (**17**) has a considerable effect on K_i and also shows how sensitive this region can be to substitution. Small, rather hydrophobic residues (e.g., in **16**) are better tolerated in the 5′ position in the active site of the kinase.

Effect of Modification of Adenine Position 6 on Inhibition of Rhodopsin Kinase. As mentioned previously, rhodopsin kinase prefers ATP over GTP as a donor of phosphate. Since both nucleotides differ in positions 2 (which is less restricted) and 6, we examined the effect of systematic changes at position 6 (Table II). None of the analogues examined that had hydrophobic or bulky substituents in position 6 (**20**–**24**) were good inhibitors. In addition, none of the conservative substitutions (**18**, **19**) yielded effective inhibitors. This position may be important in interaction with acidic residues of the kinase [because there is no inhibition at all when the NH_2 is protonated, as in **20** or **25** (Kusachi et al., 1985)]. Since substitutions in position 6 do not yield effective inhibitors, analogues based on these compounds would be expected to be poor choices for affinity purification of the rhodopsin kinase.

Effect of Base-Modified Nucleotides on Inhibition of Rhodopsin Kinase. Systematic screening of adenosine analogues in position X_7 and Y_9 appears to be very successful in finding a family of competitive inhibitors with respect to ATP for rhodopsin kinase (Table III). Both adenosine (**8**), an *N*-glycoside, and tubercidin 5′-phosphate (**26**), a *C*-glycoside, are good inhibitors (Table III) with similar K_i's (4.0 and 3.6 μM, respectively). The substitution in position X_7 that converts the purine ring to a pyrrolopyrimidine (as in **27**) leads to a 10-fold decrease in K_i. This compound, formycin monophosphate (**27**), can still be a very useful analogue in studies of the active site of rhodopsin kinase due to its fluorescent properties; the more popularly used ethenoadenosine does not bind to the kinase.

Table III: Kinetic Parameters for Base-Modified Nucleosides

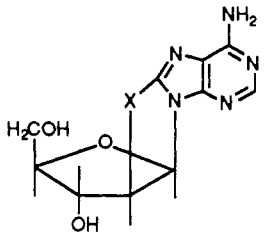


no.	X_7	Y_9	$Z_{5'}$	K_i (μM)
8	N	N	CH_2OH	4.0
26	N	CH	CH_2P_i	3.6
27[a]	CH	N	CH_2P_i	36
28	$C-C(=O)NH_2$	N	CH_2OH	0.18
29	$C-C(=^+NH_2)NH_2$	N	CH_2OH	2.2
30	$C-C(=NOH)NH_2$	N	CH_2OH	1.0
31	C—C≡N	N	CH_2OH	0.54

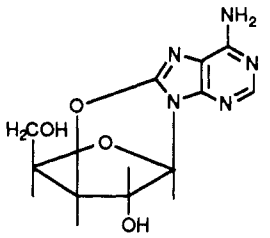
[a] N in 8 position.

Recently, the naturally occurring pyrrolopyrimidine antibiotic sangivamycin (**28**) was tested as an inhibitor of various protein kinases (Saffer & Glazer, 1981; Loomis & Bell, 1988). The K_i's were in the range 10–100 μM but were even higher for the cAMP-dependent protein kinase and β-adrenergic receptor kinase (Loomis & Bell, 1988; J. Benovic, personal communication, 1989). This last kinase has a special interest

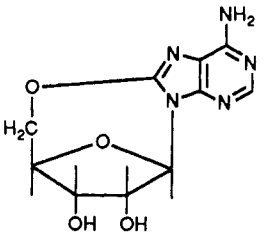
Table IV: Kinetic Parameters for Conformationally Restricted Nucleosides



8,2′-cyclo



8,3′-cyclo



8,5′-cyclo

no.	compound	K_i (μM)	X
8	adenosine	4.0	
32	8,2′-anhydro-8-mercapto-9-(β-D-arabinofuranosyl)adenine	520	S
33	8,2′-anhydro-8-oxy-9-(β-D-arabinofuranosyl)adenine	no effect	O
34	8,3′-anhydro-8-oxy-9-(β-D-xylofuranosyl)adenine	10	
35	8,5′-anhydro-8-oxy-9-(β-D-ribofuranosyl)adenine	27	

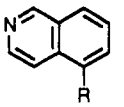
for us, since many properties of β-adrenergic kinase are similar to those of rhodopsin kinase (Benovic et al., 1987b, 1989b; Hargrave et al., 1988; Palczewski et al., 1988a,b, 1989). The much lower K_i of sangivamycin (**28**) for rhodopsin kinase (180 nM) than for β-adrenergic receptor kinase (IC_{50} = 67 μM; J. Benovic, personal communication) may be used as a tool to discriminate among members of this class of receptor protein kinases. All X_7 analogues with *potential* H-bond acceptor groups in this position (**28–31**) are extremely good competitive inhibitors of rhodopsin kinase.

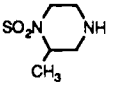
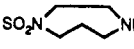
Inhibition of Rhodopsin Kinase by Conformationally Restricted Nucleosides. The purine portion of ATP must be bound by the active site of rhodopsin kinase in a very specific way (Tables I–III). From the experiments described above, it appears that the most likely conformation torsion angle between ribose and base for an effective inhibitor of rhodopsin kinase is a conformation intermediate between syn and anti. In order to test directly which conformation best fits the enzyme binding site, kinetic parameters of four nucleosides with restricted conformation (**32–35**) were measured (Table IV). The 8,3′-cyclonucleoside **34** is a potent inhibitor of rhodopsin kinase, suggesting that the conformation of the ATP that is bound by the kinase resembles that of this constrained analogue. The 8,5′-cycloadenosine **35** is also a good inhibitor of rhodopsin kinase; however, its K_i is slightly higher than that of the 8,3′-cyclo analogue (27 μM vs 10 μM, respectively). On the other hand, the 8,2′-cyclonucleosides **32** and **33** are very poor inhibitors. It is unknown whether this lack of inhibition is due to the special conformation of these nucleosides or the absence of the important 2′OH group of the carbohydrate moiety (Palczewski et al., 1988b).

Effect of Isoquinolinesulfonamide Derivatives on Rhodopsin Kinase Activity. It was of interest to test whether widely used inhibitors of protein kinases such as isoquinoline derivatives (**36–38**; Hidaka et al., 1984), a naphthalene derivative (**39**; Hidaka et al., 1984), and a benzimidazole derivative (**40**; Zandomeni & Weinmann, 1984) are inhibitors of rhodopsin kinase.

As shown in Table V, all isoquinoline derivatives tested (**36–38**) are inhibitors of rhodopsin phosphorylation; however, the K_i's are about 1 order of magnitude higher than those for other tested protein kinases. Similarly, for β-adrenergic receptor kinase these analogues are not potent inhibitors (Benovic et al., 1989a). Thus, proper application of these inhibitors should allow preferential inhibition of other protein kinases with only a minimal effect on rhodopsin kinase and will nicely complement sangivamycin, which can be applied with the opposite effect. Compound **39** is not an inhibitor of rhodopsin

Table V: Isoquinolinesulfonamide and Other Derivatives as Inhibitors of Rhodopsin Kinase



no.	compound	R	K_i (μM)
36	H 7		46
37	H 8	$SO_2NH(CH_2)_2NHCH_3$	58
38	HA 1004	$SO_2NH(CH_2)_2NHC(=NH)NH_2$	51
39	M1-9[a]		no effect
40	5,6-dichloro-1-(β-ribofuranosyl)-benzimidazole (DRB)		4.0

[a] Naphthalene ring instead of isoquinoline.

kinase, whereas the benzimidazole derivative (**40**) is as potent an inhibitor as adenosine (K_i = 4 μM). It has been reported that **40** inhibits casein kinase II activity (Zandomeni & Weinmann, 1984). However, low solubility of this derivative restricts its application.

Can Sangivamycin Block Rhodopsin Kinase Activity in ROS? It was important to test whether sangivamycin could block rhodopsin kinase activity not only in a purified system but in whole ROS, where other proteins are present. As shown in Figure 1, sangivamycin is a very potent inhibitor of rhodopsin phosphorylation with I_{50} = 4.5 μM in the presence of 200 μM ATP. This opens the possibility of applying this analogue in more intact systems to block rhodopsin kinase activity.

Conformation of Nucleosides and Nucleotides in Solution. The relative populations of the syn and anti conformations of purine nucleosides and nucleotides can be determined by comparing the relative magnitudes of the NOE's between the 8H proton on the purine base and the 1′H and 2′H protons on the sugar ring. Computer modeling indicates that the 1′H approaches within 2.49 Å of the 8H proton in the syn conformation. In the anti conformation, the 8H proton comes within 2.79 Å of the 2′H proton. Because of various competing relaxation pathways for these and other protons, and also because of the relative ease of rotation about the glycosyl $C_{1'}$–N linkage, quantitative analysis of the NOE's of nucleosides is quite involved and is model dependent (Schirmer et al., 1972). Fortunately, adenosine (**8**) has been extensively studied and has been shown to have roughly equal (within a

Table VI: ^{1}H NMR Chemical Shifts of the Nucleosides Used in the Nuclear Overhauser Experiments[a]

no.	nucleoside	chemical shifts (ppm) 8H	2H	1'H	2'H	3'H	4'H	5'H/5''H
8	adenosine	8.31	8.2	6.07	4.72	4.36	4.23	3.82
14	8-bromoadenosine		8.25	6.2	5.11	4.53	3.37	3.95
15	2-chloroadenosine	8.26		5.96	4.75	4.43	4.3	3.85
16	5'-(*N*-ethylcarbamoyl)adenosine	8.19	8.15	6.1	4.91	4.7	3.32	1.1
28	sangivamycin	8.25	8.32	6.25	4.6	4.37	4.27	3.88
31	toyocamycin	8.17	8.25	6.17	4.6	4.3	4.2	3.8

[a] Spectra were collected at pH 7.0 in 99% D_2O at 25 °C. Chemical shifts are referenced to the sodium salt of 3-(trimethylsilyl)propionic-*2,2',3,3'-d*$_4$ acid.

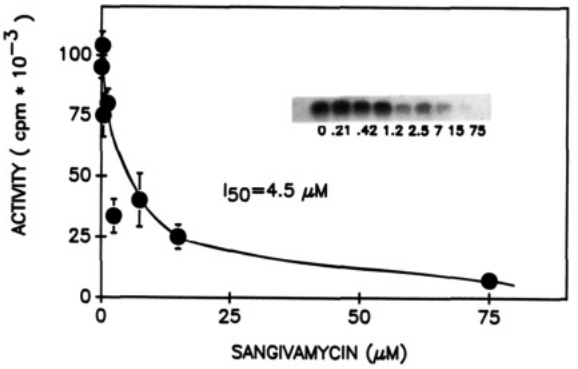


FIGURE 1: Inhibition of rhodopsin phosphorylation in whole ROS by sangivamycin. ROS (90 μL) were suspended in 70 mM potassium phosphate buffer containing 1 mM $MgCl_2$, 0.1 mM EDTA, and 200 μM [γ-^{32}P]ATP (100 cpm/pmol) to a final concentration of rhodopsin of 2.15 mg/mL. Identical samples were mixed with 10-μL aliquots of different concentrations of sangivamycin in the above buffer, and the samples were illuminated in a water bath at 30 °C (150-W flood lamp at 10 cm). After 10 min, phosphorylation was stopped by adding 50 μL of 10% SDS in electrophoresis buffer (Laemmli, 1970). SDS–PAGE followed by staining and autoradiography were performed. Radioactive bands containing rhodopsin were cut out and dissolved in H_2O_2. The extent of phosphorylation was determined by measurement of radioactivity. Inset: Autoradiogram of rhodopsin band in the sample containing 0, 0.21, 0.42, 1.2, 2.5, 7, 15, or 75 μM sangivamycin.

factor of 3 or 4) populations of syn and anti conformers (Davies, 1978). Using the NOE's measured for adenosine as indicators of nearly syn and anti conformers, we can obtain a qualitative indication of the preferences of the other nucleosides for syn and anti conformations by noting the relative magnitudes of $f_8(1')$ and $f_8(2')$ (Tables VI and VII). Thus, in going from adenosine (**8**) to toyocamycin (**31**), $f_8(1')$ and $f_8(2')$ change dramatically from 9 and 0.6, respectively, to 4 and 9 (Table VII). This can be taken as evidence for a preference for the anti conformation in toyocamycin. Similar results are observed for sangivamycin. In contrast, the relative magnitudes of the two NOE's for 2-chloroadenosine (**15**) are essentially the same as those observed for adenosine, indicating that there is little preference for either the syn or anti conformation in 2-chloroadenosine (Table IV).

Unfortunately, the absence of the 8H proton in 8-bromo-AMP (**13**) and 8-bromoadenosine (**14**) does not permit this type of analysis. However, the effect of bromine substitution has been extensively studied and it is known that the syn conformation is highly preferred in these compounds (Sarma et al., 1974).

Conclusion

On the basis of the substrate specificity and competition with ATP of nucleosides and nucleotides, the following requirements appear to be important for nucleoside binding to the active site of rhodopsin kinase: (1) The N-7 and N^6 nitrogens of the purine ring are essential for binding at the active site. (2) The 2'OH group of the ribose moiety is involved in interaction with the enzyme at the active site (Palczewski et al., 1988b). (3) Positions 2 and 8 of the purine ring play a minor role in enzyme substrate recognition. (4) The anti conformation of the nucleoside, or a conformation intermediate between syn and anti, is preferred. From these studies, we can conclude that only nucleotide (nucleoside) ligands attached through the ribose 3' or purine 2 position may be useful for affinity chromatography of rhodopsin kinase.

Table VII: Summary of NOE Results[a]

no.	compound	$f_8(1')$	$f_8(2')$	conformation	ref
8	adenosine	9	0.6	syn/anti	*b*
13	8-bromo-AMP	nd	nd	syn	*c*
14	8-bromoadenosine	nd	nd	syn	*c*
15	2-chloroadenosine	12	2	syn/anti	
16	5'-(*N*-ethylcarbamoyl)adenosine	13	12	anti > syn	
28	sangivamycin	6	13	anti	
31	toyocamycin	4	9	anti	

[a] All samples were at pH 7.0 except for sangivamycin, which was at pH 3.2. Concentrations were as follows: 2-chloroadenosine, 5.2 mM; 5'-(*N*-ethylcarbamoyl)adenosine, 2.5 mM; adenosine, 4.6 mM; toyocamycin, 6.1 mM; sangivamycin, 0.75 mM. [b] From Gueron et al. (1973). [c] From Sarma et al. (1974).

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