ACTIVITY OF TUBERCIDIN-, TOYOCOMYCIN-, AND SANGIVAMYCIN-3',5'-CYCLIC PHOSPHATES AND RELATED COMPOUNDS WITH SOME ENZYMES OF ADENOSINE-3',5'-CYCLIC PHOSPHATE METABOLISM

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SUMMARY

The effect of modifying adenosine 3',5'-cyclic phosphate in the 7-position was studied by investigating tubercidin-, toyocomycin-, and sangivamycin-3',5'-cyclic phosphates as activators of adenosine 3',5'-cyclic phosphate-dependent protein kinases and as substrates for and inhibitors of adenosine 3',5'-cyclic phosphate phosphodiesterases. The same decreasing order of activity was seen with the cyclic phosphates as kinase activators or phosphodiesterase substrates: tubercidin- > toyocomycin- > sangivamycin-3',5'-cyclic phosphate. The I_{50} values of the heterocyclic base, nucleoside, nucleoside 5'-phosphate, and nucleoside 3',5'-cyclic phosphate of tubercidin, toyocomycin, and sangivamycin were determined for the inhibition of adenosine 3',5'-cyclic phosphate hydrolysis. The heterocyclic bases and the nucleoside 3',5'-cyclic phosphates were very good inhibitors while the nucleosides and nucleoside 5'-phosphates were in general quite poor inhibitors.

INTRODUCTION

In a series of recent papers, we have investigated the effects of various modifications of adenosine 3',5'-cyclic phosphate (cAMP) in the heterocyclic (1-3), carbohydrate (4), and cyclic phosphate (5,6) moieties on the biological activity of the resulting compounds. The report of the synthesis of tubercidin 3',5'-cyclic phosphate (7) which mimicked several of the biological activities of cAMP (8-11), suggested a study into a series of other modifications of cAMP in the 7-position. We report here the synthesis of toyocomycin 3',5'-cyclic phosphate (8), and sangivamycin 3',5'-cyclic phosphate (12), and compare their enzymic properties with those of tubercidin 3',5'-cyclic phosphate (4) and their respective heterocyclic bases, nucleosides and 5-nucleotides.

METHODS

Table 1 gives the structure of the compounds investigated. 4-Amino-

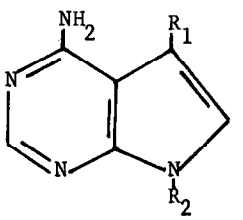
pyrrolo[2,3-d]pyrimidine (1) and 4-aminopyrrolo[2,3-d]pyrimidin-5-carbonitrile (5) were generous gifts from Dr. Leroy B. Townsend. Tubercidin 3',5'-cyclic phosphate (4) was a generous gift from Dr. Paul O'Connell of the Upjohn Co., Kalamazoo, Michigan. Tubercidin (2), toyocomycin (6) and sangivamycin (10) were synthesized by the procedure of Tolman et al. (12). The corresponding nucleotides 3, 7 and 11 were prepared by the general procedure of Yoshikawa et al. (13), and were purified by Dowex 1 (formate) chromatography. Toyocomycin 3',5'-cyclic phosphate (8) was prepared from 7 by the general procedure of Smith et al. (14) with the modification of pyridine/N,N-dimethylformamide (1/1) for the reaction solvent. Sangivamycin 3',5'-cyclic phosphate (12) and 4-aminopyrrolo[2,3-d]pyrimidine-5-carboxamide (9) [uv: $\lambda_{max}^{pH\ 1}$ 274 nm (ϵ28,000), $\lambda_{max}^{pH\ 11}$ 278 nm (ϵ29,400)] were prepared from 8 and 5, respectively, by the general procedure as described by Tolman et al. (12). The newly synthesized compounds 3, 7, 8, 9, 11 and 12 were verified for purity and structure by uv and pmr spectra, elemental analysis, thin-layer chromatography and where appropriate, electrophoresis.

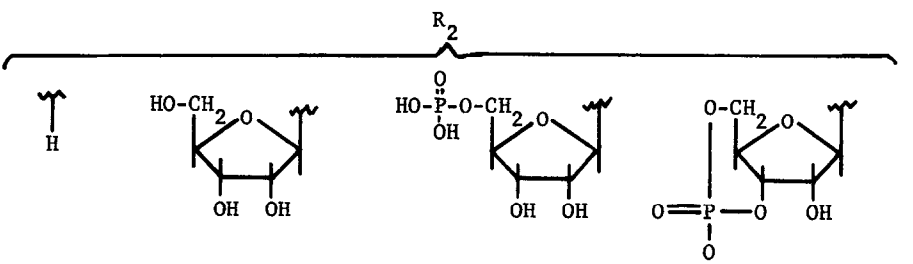
RESULTS AND DISCUSSION

Table 2 compares the ability of tubercidin- (4), toyocomycin- (8), and sangivamycin 3',5'-cyclic phosphate (12) to activate the cAMP-dependent protein kinase from beef brain and beef heart. There was little difference in the K_a' values between the two enzymes for any of the three cyclic nucleotides, whereas the three compounds demonstrated significant differences in activating activity. Tubercidin 3',5'-cyclic phosphate (4) was a significantly better activator than cAMP, while toyocomycin 3',5'-cyclic phosphate (8) was only about 1/2 as active as cAMP and sangivamycin 3',5'-cyclic phosphate (12) was only about 1/5 as active. These results show that, while the replacement of the nitrogen in the 7-position with carbon is well tolerated and actually improved the activating capability of the resulting compound, substitution of the C-7 with -CN or $-CONH_2$ appreciably diminished the activity of the resulting compound, i.e., toyocomycin 3',5'-cyclic phosphate (8) and sangivamycin 3',5'-cyclic phosphate (12).

Table 1

Chemical Structures





R_1	R_2 = H	R_2 = HO-CH$_2$ (OH, OH)	R_2 = HO-P(=O)(OH)-O-CH$_2$ (OH, OH)	R_2 = O-CH$_2$ (O=P(=O)(OH)-O, OH)
-H	1	2	3	4
-CN	5	6	7	8
-CONH$_2$	9	10	11	12

Kuo and Greengard (8) have reported a K_a' for tubercidin 3',5'-cyclic phosphate (4) with the beef brain and beef heart protein kinases of 0.69 and 1.6, respectively. These differences between their and our results may be because they used a reference value for their K_a for cAMP, while we have compared tubercidin 3',5'-cyclic phosphate (4) with cAMP in the same experiment.

The ability of these derivatives to serve as substrates for rabbit kidney and beef heart cAMP phosphodiesterases was studied and the results are summarized in Table 3. All three derivatives were hydrolyzed by both enzymes,

Table 2

Activation of cAMP-Dependent Protein Kinases by

Tubercidin-, Toyocomycin-, and Sangivamycin 3',5'-Cyclic Phosphates

Compound	K_a'	
	Beef Brain	Beef Heart
Tubercidin 3',5'-cyclic phosphate (4)	1.5	1.3
Toyocomycin 3',5'-cyclic phosphate (8)	0.52	0.44
Sangivamycin 3',5'-cyclic phosphate (12)	0.23	0.19

cAMP-dependent protein kinases were purified through the DEAE-cellulose step (12,13). The assay for the stimulation of the cAMP-dependent protein kinase contained in 0.1 ml: 5 μmol NaOAc, pH 6.0; 1 μmol $MgCl_2$; 15 μg histone (Worthington HLY); 0.5 nmol ATP-[γ-^{32}P]; protein kinase enzyme (20-200 μg); and various concentrations of the 3',5'-cyclic nucleotide being tested as an activator (10^{-9} - 10^{-3} M). The assay was performed and the K_a values determined as previously described (1). The $K_a' = \frac{(K_a \text{ for cAMP})}{K_a \text{ cyclic nucleotide derivative}}$, where the K_a values for cAMP are 1.1×10^{-7} M and 7.3×10^{-8} M for the brain and heart enzymes, respectively.

although at rates less than that for cAMP. With both enzymes, the order of activity was similar: tubercidin 3',5'-cyclic phosphate (4) > toyocomycin 3',5'-cyclic phosphate (8) > sangivamycin 3',5'-cyclic phosphate (12), the same order of activity that was seen with the protein kinases (Table 2). We have previously reported that some 5'-deoxy-5'-thioadenosine 3',5'-phosphorothioate derivatives demonstrated a similar spectrum of activities as an activator of beef brain cAMP-dependent protein kinase and as a substrate for rabbit kidney cAMP phosphodiesterase (6). The present results substantiate that these two enzymes have certain similar steric and/or electronic requirements of the substrate for interaction with their active sites.

Table 4 compares the ability of heterocyclic base, nucleoside, nucleoside 5'-phosphate, and nucleoside 3',5'-cyclic phosphate of tubercidin (2), toyocomycin (6) and sangivamycin (10) to inhibit the hydrolysis of cAMP by the

Table 3

Hydrolysis of Tubercidin-, Toyocomycin-, and Sangivamycin 3',5'-Cyclic Phosphates by cAMP Phosphodiesterases

Compound	Relative Rate of Hydrolysis	
	Beef Heart	Rabbit Kidney
Adenosine 3',5'-cyclic phosphate	1.0	1.0
Tubercidin 3',5'-cyclic phosphate (4)	0.77	0.59
Toyocomycin 3',5'-cyclic phosphate (8)	0.53	0.39
Sangivamycin 3',5'-cyclic phosphate (12)	0.29	0.16

The cAMP phosphodiesterases were purified as described previously (4). The standard reaction mixture contained in 0.60 ml: 3.0 μmol cyclic nucleotide; 30 μmol tris·HCl, pH 7.5; 6 μmol $MgCl_2$; and phosphodiesterase protein (0.15 mg of rabbit kidney enzyme and 0.22 mg of beef heart enzyme). After an appropriate incubation period (10-60 min), the reaction was terminated by heating, treated with bacterial alkaline phosphatase, and the phosphate released was assayed colorimetrically as previously described (4). The actual rates of hydrolysis of cAMP were 45 and 32 nmol 5'-AMP formed per min for the kidney and heart enzymes, respectively.

rabbit lung and beef heart cAMP phosphodiesterases. The three cyclic nucleotides were all very good inhibitors. This is probably partly because they are serving as substrates for these enzymes. The nucleosides and nucleoside 5'-phosphates were in general quite poor inhibitors of the enzymes, while the heterocyclic bases were good inhibitors, but not as active as the cyclic nucleotide. That the nucleosides and nucleoside 5'-phosphates were poor inhibitors, while the cyclic nucleotides were good inhibitors, indicates that the ribose 3',5'-cyclic phosphate moiety is also a point of interaction between the cyclic nucleotide and the cAMP phosphodiesterases. Preliminary studies on the kinetics of inhibition of the rabbit lung and beef heart phosphodiesterases have shown that the 3',5'-cyclic nucleotides (4, 8, 12), 5'-nucleotides (3, 7, 11), and nucleosides (2, 6, 10) are all competitive inhibitors of both enzymes, while the heterocyclic bases (1, 5, 9) show a mixed type of inhibition. These

Table 4

Inhibition of cAMP-Phosphodiesterases by Tubercidin, Toyocomycin, Sangivamycin, and Related Derivatives

	I_{50} (μM)											
	Chemical Form of Derivative											
	Heterocyclic Base			Nucleoside			5'-Nucleotide			3',5'-Cyclic Nucleotide		
Parent Nucleoside	Cpd. No.	Rabbit Lung	Beef Heart	Cpd. No.	Rabbit Lung	Beef Heart	Cpd. No.	Rabbit Lung	Beef Heart	Cpd. No.	Rabbit Lung	Beef Heart
Tubercidin	1	290	200	2	> 1000	> 1000	3	> 2000	170	4	12	20
Toyocomycin	5	10	29	6	220	250	7	1000	1000	8	2.4	2.0
Sangivamycin	9	45	60	10	1000	500	11	1000	1000	12	6.7	5.0

The rabbit lung and beef heart phosphodiesterases were prepared as previously described(4). The assay contained in 0.5 ml: 25 μmol tris·HCl, pH 7.5; 5 μmol $MgCl_2$; 20-200 μg phosphodiesterase protein; 80 nmol 8-[^{3}H]-cAMP (350,000 cpm); and varying concentrations of the 3',5'-cyclic nucleotide being tested as an inhibitor. The mixture was heat-inactivated to terminate the reaction, treated with 5'-nucleotidase and Dowex 1, and the radioactivity of the nucleoside fraction determined as previously described (4).

results indicate that the heterocyclic bases may be binding to the enzymes in a manner different from that of the other compounds. The analysis of these differences is underway.

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