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PYRROLO[2,3-d]PYRIMIDINES AS INHIBITORS OF cAMP-PHOSPHODIESTERASE

STRUCTURE–ACTIVITY RELATIONSHIP

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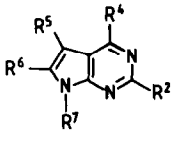
Abstract—The effects of pyrrolo[2,3-d]pyrimidine compounds (7-desazapurines) on cAMP hydrolyzing, calmodulin dependent and calmodulin independent phosphodiesterase were studied. Phosphodiesterase inhibition depended on the chemical nature of substituents attached to the pyrrolo-pyrimidine-nucleus at positions 2, 4, 5, 6 and 7. Among a total of 28 compounds tested, the 4-amino-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine-5,6-dicarbaldehyde (**9**) was the most potent inhibitor of phosphodiesterase activity ($IC_{50} = 16\ \mu M$). In addition to the 5,6-disubstitution, position 2 of the pyrrolo-pyrimidine derivatives had to be unsubstituted and position 4 had to bear an amino-group for an optimal inhibitory effect. Calmodulin dependent and calmodulin independent isozymes were affected to the same extent. Inhibition of PDE activity was reversible upon removal of the pyrrolo-pyrimidine derivative **9** and non-competitive with respect to cAMP ($K_i = 27\ \mu M$).

Pyrrolo[2,3-d]pyrimidine derivatives are found in nature as rare constituents of t-RNA in eucaryotes [1] and in culture filtrates of microorganisms [2]. Various strains of Streptomyces produce and excrete N-7 ribosylated pyrrolo[2,3-d]pyrimidine derivatives, which have potent antibiotic and antitumor activity, e.g. tubercidine, toyocamycine, and sangivamycine [2, 3]. Despite their high toxicity they are of interest as antineoplastic agents and some of them are currently undergoing clinical trials [4]. Chemically synthesized 7-phenyl-pyrrolo[2,3-d]pyrimidines reveal quite different pharmacological effects: analgesic, sedative, antiphlogistic, and anti-inflammatory [5, 6].



1-28

Scheme 1

Although pyrrolo[2,3-d]pyrimidines have recently been reported to inhibit lipoxygenase (with low potency) [7] and to be adenosine antagonists at A1- and A2-adenosine receptors (with high potency) *in vitro* [8], the molecular mechanism by which the pyrrolo[2,3-d]pyrimidines exert their diverse pharmacological effects *in vivo*, remains to be elucidated. As a further step towards this goal, we studied the effect of the pyrrolo[2,3-d]pyrimidines on cAMP-PDE†, since (i) cyclic nucleotides may play an important role in inflammatory reactions [9], and (ii) some antidepressant and other psychotropic drugs act as PDE inhibitors [10, 11]. Furthermore, the chemical structure of the pyrrolo[2,3-d]pyrimidines prompted us to screen for potential substrate antagonist activity on cAMP-PDE.

In this study 28 pyrrolo[2,3-d]pyrimidines were tested for their effects on calmodulin dependent and independent cAMP-PDE activity. Variations in the substitution pattern of the pyrrole- and the pyrimidine-moieties, respectively, led to differences in inhibitory potency. The most potent PDE inhibitor **9** was comparable to IBMX, a PDE inhibitor commonly used in laboratories.

MATERIALS AND METHODS

Pyrrolo[2,3-d]pyrimidines

The synthesis of compounds (see Table 1) **1, 25** [12]; **6–8** [13]; **2, 3, 12–18, 21, 22** [5]; **9–11** [8]; **23, 24, 27** [14] has been described.

Compounds **19** (mp 228°), **20** (mp 259°), **26** (mp dec 118°), **28** (mp 125–127°) were prepared in analogy to the method published before [14]. Procedures for the synthesis of compounds **4** and **5** are provided below. Melting points were determined in open capillaries and are uncorrected. The ^{1}H-NMR spectra were recorded on a Bruker-AC 80 with TMS as internal standard and [$^2H_6$]ME_2SO as solvent. All compounds analysed correctly for carbon, hydrogen and nitrogen. Prior to being tested, the purity of all the compounds was assured by ^{1}H-NMR spectroscopy and thin-layer chromatography.

4-Hydrazino-5,6-dimethyl-7-phenyl-7H-pyrrolo-[2,3-d]pyrimidine (**5**). The intermediate 4-chlor-5,6-dimethyl-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine (mp 162°) was synthesized according to the method described before [12]. ^{1}H-NMR; $\delta =$

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† Abbreviations: PDE, phosphodiesterase (EC 3.1.4.17); IBMX, 3-isobutyl-1-methylxanthine.

2.2(s,3H,6-CH_3), 2.45(s,3H,5-CH_3), 7.5(m,5H, arom.), 8.45(s,1H,2-H).

4-Chlor-5,6-dimethyl-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine (0.6 g, 2.3 mmol) and anhydrous hydrazine (5 g) were refluxed in ethanol (20 g) for 6 hr. After cooling compound **5** (mp 155°–157°) crystallized from the reaction mixture in 84.7% yield. ^{1}H-NMR; δ = 2.2(s,3H,6-CH_3), 2.45(s,3H,5-CH_3), 3.9(s br,3H,NH-NH_2), 7.5(m,5H,arom), 8.15(s,1H, 2-H).

5,6-Dimethyl-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine (4)

A solution of potassium hydroxide (1.0 g) in ethanol (20 g) was added with stirring to a solution of **5** (1.5 g, 5.9 mmol) in ethanol (40 g). Within 1 min the mixture changed its colour to dark blue. It was stirred at room temperature for 12 hr, then the solvent was removed *in vacuo*. Water was added to the residue and after filtration the precipitate was recrystallized from ethanol to give **4** (mp 90°) in 75.6% yield. ^{1}H-NMR: δ = 2.25(s,3H,6-CH_3), 2.3(s,3H,5-CH_3), 7.5(m,5H,arom.), 8.6(s,1H,2-H), 8.9(s,1H,4-H).

Proteins

Calmodulin dependent cAMP-PDE and calmodulin were purified to homogeneity from bovine brain using calmodulin affinity- and phenyl sepharose chromatography as the final purification steps, respectively [15–17]. Protein was determined by the method of Lowry using bovine serum albumin as a standard.

Phosphodiesterase assay

Enzyme activity was measured by the method of Pöch [18]. Incubations at 37° in a total volume of 500 μl contained 10 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM AMP, 0.1 mM $CaCl_2$, 20 μM cAMP including 20 nCi ^{3}H-cAMP and either 3.3 μg cAMP-specific calmodulin independent bovine heart PDE obtained from Boehringer, Mannheim, or 5 μg calmodulin dependent PDE plus 0.1 μg calmodulin. All assays were done in duplicate. Stock solutions (5 mM) of the pyrrolo[2,3-d]pyrimidine derivatives were prepared in a solution of glucose (30%, w/w). Sometimes sonication was necessary to achieve dissolution. The enzyme reaction was terminated after 20 min by sequential addition of $ZnSO_4$ and $Ba(OH)_2$. AMP was coprecipitated with $BaSO_4$ and separated by centrifugation of the samples. An aliquot of the supernatant containing the remainder of the substrate was removed and its radioactivity was measured. The assay was within the linear range of time and protein content. Activity of 100% represented the hydrolysis of 56 and 42 nmol cAMP/min $\times$ mg^{-1} for the calmodulin independent and calmodulin dependent form of cAMP-PDE, respectively.

RESULTS AND DISCUSSION

Structure–activity relationship

The present series of pyrrolo[2,3-d]pyrimidines represents a little-examined class of PDE inhibitors [19–21]. Their potency varied with the chemical alteration of the molecule. Therefore we systematically explored the effect on PDE activity of pyrrolo[2,3-d]pyrimidine derivatives modified in positions 2 (Fig. 1A), 4 (Fig. 1B), 5, 6 and 7 (Table 1).

First, a number of substitutions was carried out at N-7 of the 4-amino-5,6-dimethylpyrrolo[2,3-d]pyrimidine (**1**). Introduction of a phenyl ring **2** led to a 16.5-fold increase in PDE inhibition compared to the N-7 unsubstituted compound **1**. This effect encouraged us to vary the lipophilicity and electronic character of the phenyl ring according to the Topliss scheme [22]. Because the substitution with chlorine (**12**) or a methoxy group (**13**) in the *para*-position of the phenyl moiety decreased the inhibitory activity of the compounds, we introduced a chlorine to the *meta*-position (**14**). However, this did not enhance the inhibitory potency. Since the procedure with the Topliss scheme obviously was not successful in optimizing **2**, we included the Craig diagram [23] as another method of systematically altering the phenyl ring substitution. According to this diagram halides (see **12, 14, 15**), a nitro group (see **18**), a dimethylamino group (see **19**), a methoxy group (see **13, 17**), and a propionyl group (see **20**), respectively, were introduced into the phenyl ring. However, none of these substituents could improve the PDE inhibitory potency of **2**. Substitution in the *para*-position (see **12, 13, 15, 18–20**) resulted in an almost complete loss of activity and *meta* substitution (**14, 17**) led to about 3-fold reduction of inhibition compared to the phenyl ring. This indicates the existence of a snug and lipophilic hole at the binding-site for pyrrolo[2,3-d]pyrimidines on the PDE, where the phenyl ring fits exactly. Interestingly, *m,p*- (**21**) and *m,m'*-dimethoxy substitution (**22**), respectively, of the phenyl ring did not cause as big a loss of activity as would be expected. Therefore steric reasons cannot completely explain the different inhibitory potencies of the N-7 substituted pyrrolo[2,3-d]pyrimidines.

Since the various substituents at the phenyl ring only decreased the inhibitory potency of the pyrrolo[2,3,-d]pyrimidines, we turned to other modifications. The distance between the heterocyclic nucleus and the phenyl ring was enlarged by a methylene group (**23**). This also decreased the inhibitory effect to the level seen with the *meta* substituted phenyl ring.

The effect of introducing saturated aliphatic groups was examined next. Attachment of a cyclopentyl- (**25**) or a cyclohexylgroup (**24**) to N-7 made the pyrrolo[2,3-d]pyrimidine derivatives more potent than **1**, though the potency of the phenyl ring substituted compound **2** was not reached. Introducing saturated aliphatic chains with four (**26**), six (**27**) or eight (**28**) C-atoms revealed that among those tested a length of six C-atoms was optimal for PDE inhibition. The half-maximal effect was about the same as for the phenyl ring substituted derivative **2**.

The 4-amino-5,6-dimethyl-7-phenyl-pyrrolo[2,3-d]pyrimidine **2** was taken for further derivatization at positions 2 (Fig. 1A), 4 (Fig. 1B), 5 and 6, because of its chemical stability, its ease of synthesis and its good PDE inhibitory activity. It was found to be essential for PDE inhibition that position 2 (Fig. 1A) of **2** remained unsubstituted. Methylation in this position (**7**) shifted IC_{50} from 60 μM to 600 μM. Other modifications (**6, 8**) even resulted in a complete loss

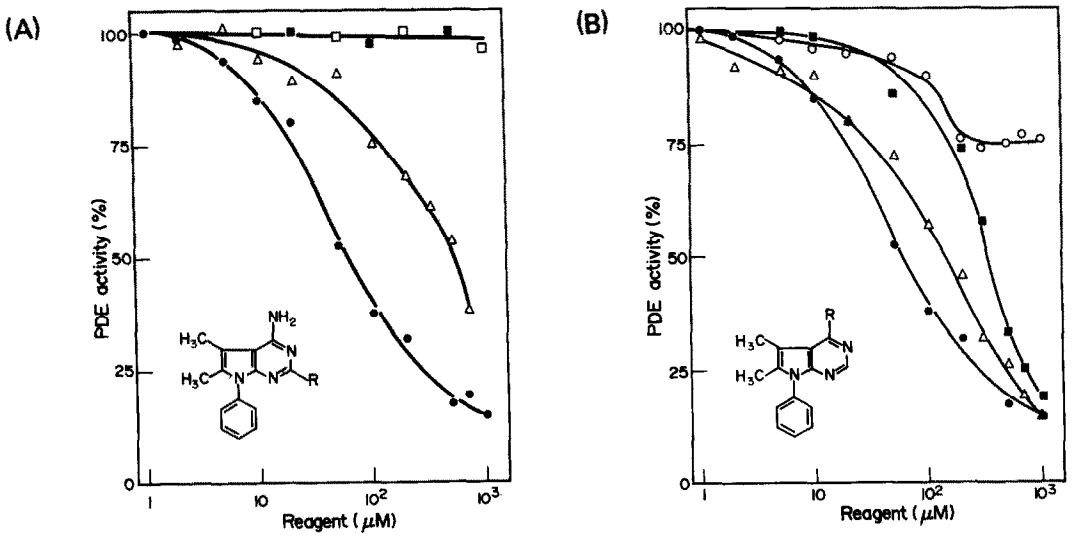

Fig. 1. Inhibition of cAMP-hydrolyzing calmodulin independent PDE by pyrrolo[2,3-d]pyrimidine derivatives. Enzyme activity of 100% corresponded to the hydrolysis of 56 nmol cAMP/min × mg^{-1}. (A) Substituents of 4-amino-5,6-dimethyl-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine at position 2 were -H (●), $-CH_3$ (△), $-NH_2$ (□), and $-C_2H_5$ (■). (B) Substituents of 5,6-dimethyl-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine at position 4 were -H (△), $-NH_2$ (●), $NH-NH_2$ (■), and -OH (○).

Table 1. Potencies of pyrrolo[2,3-d]pyrimidine derivatives as PDE inhibitors

No.	Compound R^2	R^4	R^5,R^6	R^7	IC_{50} (μM)
1	H	NH_2	CH_3	H	1000
2	H	NH_2	CH_3	C_6H_5	60
3	H	OH	CH_3	C_6H_5	20%*
4	H	H	CH_3	C_6H_5	130
5	H	$NH–NH_2$	CH_3	C_6H_5	350
6	NH_2	NH_2	CH_3	C_6H_5	20%*
7	CH_3	NH_2	CH_3	C_6H_5	600
8	C_6H_5	NH_2	CH_3	C_6H_5	0%*
9	H	NH_2	CHO	C_6H_5	16
10	H	NH_2	COOH	C_6H_5	65
11	H	NH_2	H	C_6H_5	270
12	H	NH_2	CH_3	*p*-Cl-C_6H_4	600
13	H	NH_2	CH_3	*p*-OCH_3-C_6H_4	40%*
14	H	NH_2	CH_3	*m*-Cl-C_6H_4	200
15	H	NH_2	CH_3	*p*-Br-C_6H_4	20%*
16	H	NH_2	CH_3	o,*p*-diBr-C_6H_3	500
17	H	NH_2	CH_3	*m*-OCH_3-C_6H_4	250
18	H	NH_2	CH_3	*p*-NO_2-C_6H_4	30%*
19	H	NH_2	CH_3	*p*-$N(CH_3)_2$-C_6H_4	1000
20	H	NH_2	CH_3	*p*-COC_2H_5-C_6H_4	500
21	H	NH_2	CH_3	*m*,*p*-diOCH_3-C_6H_3	90
22	H	NH_2	CH_3	*m*,*m*′-diOCH_3-C_6H_3	100
23	H	NH_2	CH_3	CH_2-C_6H_5	260
24	H	NH_2	CH_3	C_6H_{11} (Cyclohexyl)	200
25	H	NH_2	CH_3	C_5H_9 (Cyclopentyl)	100
26	H	NH_2	CH_3	*n*-C_4H_9	220
27	H	NH_2	CH_3	*n*-C_6H_{13}	40
28	H	NH_2	CH_3	*n*-C_8H_{17}	5%*

* Maximal inhibition of PDE at the concentration of 1000 μM of the pyrrolo[2,3-d]pyrimidine derivative.

of the PDE inhibitory effect.

Alteration of the pyrrolo[2,3-d]pyrimidines in position 4 (Fig. 1B) revealed that the adenosine-analog **2** met the requirements for PDE inhibition best. Little effect was observed in the presence of the oxo-compound **3**. Two hundred micromoles of this drug inhibited PDE by only 25%. Higher concentrations had no further effect. This, however, may be due to the limited solubility of **3**. The unsubstituted drug **4** was half as potent as **2**, whereas the bulky and more basic hydrazinogroup **5** increased the IC_{50} from 60 μM to 350 μM. These observations are in line with the reported diminution of the biological activity of the nucleoside antibiotics by exchanging the amino group with other substituents [4].

Finally, positions 5 and 6 of **2** were varied by oxidation in analogy to potential metabolism. The resulting compounds showed quite different potency. The 5,6 unsubstituted drug **11** had only little effect on PDE activity, whereas inhibition was maximal (IC_{50} = 16 μM) with **9** being formylated in both positions. Further oxidation of the dicarbaldehyde **9** to the dicarboxylic acid **10** reduced inhibition to the level seen with the methyl groups.

Characterization of the PDE inhibition

4-Amino-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine-dicarbaldehyde **9** was the most potent PDE inhibitor found among a total of 28 compounds tested. Therefore **9** was selected for further biochemical studies on the mode of action. The efficiency of that drug (IC_{50} = 16 μM, Fig. 2) favorably compared with PDE inhibitors commonly used, e.g. IBMX. For the latter an IC_{50} value of 20 μM was obtained using the standard assay outlined above.

To study whether inhibition of PDE by pyrrolo[2,3-d]pyrimidine derivatives is a reversible process, 100 μM of **9** were added to 250 μl of buffer containing 50 mM Tris–HCl (pH 7.0), 3 mM $MgSO_4$, and PDE (2 mg/ml). The mixture was kept on ice for 1 hr. Then the material was passed over a Superose 12 gel filtration column. Virtually all enzyme activity was recovered as the inhibitor was removed. Thus, inhibition of PDE by pyrrolo[2,3-d]pyrimidines is a reversible process. This argues against covalent binding of **9** to catalytically essential groups of the protein.

The potential antidepressant rolipram ((4-(3-cyclopentyloxy)-4-methoxyphenyl)-2-pyrrolidone) was reported to specifically inhibit the calmodulin independent species of cAMP-PDE from rat and bovine brain [24, 25]. This prompted us to study PDE isozyme specificity of the pyrrolo[2,3-d]pyrimidine derivatives. Calmodulin dependent and calmodulin independent PDE were affected similarly by these compounds. Sixteen micromoles of **9** reduced both types of cAMP-PDE activity by 50% (Fig. 2). None out of 7 derivatives tested showed a significant difference in PDE inhibitory potency for the calmodulin dependent and the calmodulin independent enzyme. It is concluded, that pyrrolo[2,3-d]pyrimidine derivatives do not act as calmodulin antagonists. Further studies with individual purified isozymes will be necessary to study inhibitor specificity.

The question was addressed as to whether these heterocycles exert their effect as inhibitors of cAMP-PDE due to being 7-desazapurines. If the heterocycles are acting as substrate analogs, inhibition should be competitive with regard to cAMP. Kinetics were studied in detail using 7 μM and 21 μM of **9**, respectively. cAMP concentrations were varied from 0.1 μM to 200 μM but for technical reasons data are shown for cAMP concentrations from 5 μM to 100 μM only (Fig. 3). Plotting 1/velocity versus inhibitor concentrations (Dixon plot, Fig. 3) indicated non-competitive inhibition. The inflection point on the abscissa revealed a K_i value of 27 μM.

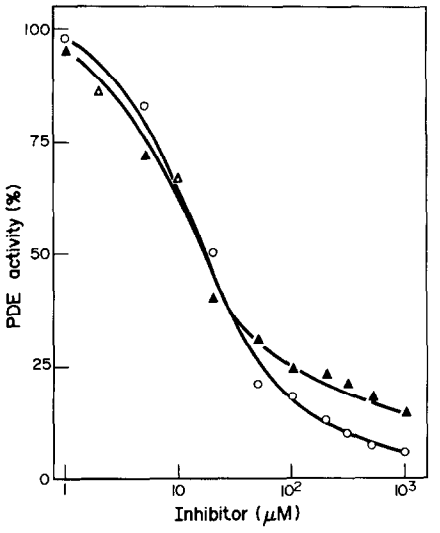

Fig. 2. Inhibition of calmodulin dependent (○) and calmodulin independent (▲) cAMP-PDE by 4-amino-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine-5,6-dicarbaldehyde (**9**). Experimental details as outlined in the Method section.

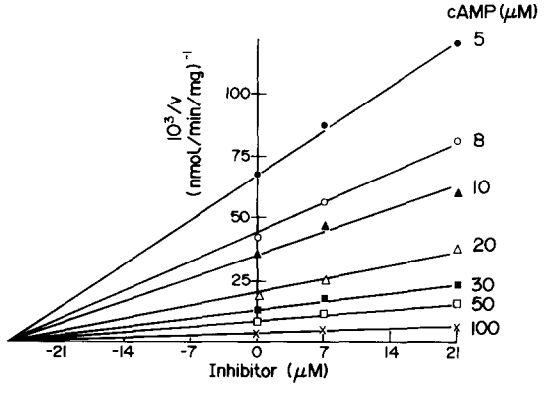

Fig. 3. Dixon plot. Kinetic analysis of the inhibition of cAMP-PDE by 4-amino-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine-5,6-dicarbaldehyde **9**. Assays were run with 3.3 μg of calmodulin independent PDE under standard conditions except for different inhibitor and substrate concentrations as indicated. cAMP was varied from 0.1 μM to 200 μM but for clarity reasons only the cAMP concentration range from 5 μM to 100 μM is shown here. A K_i of 27 μM of the inhibitor is obtained for both isozymes. Experimental details as outlined in the Method section.

Thus, PDE is inhibited by pyrrolo[2,3-d]pyrimidine derivatives in a reversible, non-competitive manner: substrate and inhibitor are mutually exclusive. We can not distinguish, however, whether the enzyme–substrate–inhibitor complex is catalytically inactive or whether the inhibitor hinders substrate binding.

In conclusion, it can be stated that in this run the 4-amino-7-phenyl-7H-pyrrolo[2,3-d]pyrimidine-5,6-dicarbaldehyde **9** is the most potent inhibitor of calmodulin independent and calmodulin dependent PDE. Neither position 2 (see **6, 7, 8**) nor position 4 (see **3, 4, 5**) may be varied without loss of activity. In position 7 only substituents with a certain volume (see **2, 27**) seemed to be allowed. Nevertheless, the influence of *o*-substitution and multiple substitution of the phenyl ring, respectively, demands further investigation (see **2, 21, 22**).

Moreover, we plan to synthesize 4-amino-7-hexyl-7H - pyrrolo[2,3 - d]pyrimidine - 5,6 - dicarbaldehyde, a combination of the dicarbaldehyde **9** and the 7-*n*-hexyl derivative **27**, as a new lead structure, since **9** and **27**, respectively, were the most potent PDE inhibitors in this study. We expect to find enhanced PDE inhibitory potency with this compound. Furthermore, it remains to be elucidated, whether positions 5 and 6, respectively, are of the same importance for PDE inhibition or not.

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