Phosphorylation of Coformycin and 2′-Deoxycoformycin, and Substrate and Inhibitor Properties of the Nucleosides and Nucleotides in Several Enzyme Systems

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Under conditions where 2′-deoxycoformycin is enzymatically phosphorylated by wheat shoot phosphotransferase to the 5′-phosphate in 15–20% yield, coformycin is a relatively poor substrate, and is phosphorylated only to the extent of ≤ 5%. However, chemical phosphorylation of coformycin by modifications of the Yoshikawa procedure led to isolation of coformycin-5′-phosphate in 20% overall yield.

Coformycin-5′-phosphate was characterized by various criteria, including ^{1}H NMR spectroscopy. Comparison of the spectrum with that of the parent nucleoside indicated that the nucleotide is predominantly, although not exclusively, in the conformation *anti* about the glycosidic bond.

Like 2′-deoxycoformycin-5′-phosphate, coformycin-5′-phosphate was a feeble substrate of snake venom 5′-nucleotidase, and is hydrolyzed, quantitatively, at only 2% the rate for 5′-AMP. With 5′-AMP analogues as substrate, the 5′-phosphates of both coformycin and deoxycoformycin were poor inhibitors of the enzyme, with K_i values > 0.3 mM.

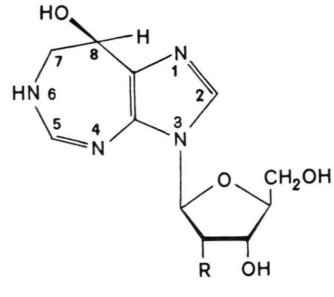
The 5′-phosphates of both coformycin and deoxycoformycin do not significantly inhibit adenosine deaminase (K_i > 0.2 mM), but are potent inhibitors of adenylate deaminase ($K_i \leq 10^{-9}$ M). Neither coformycin nor deoxycoformycin are inhibitors of mammalian purine nucleoside phosphorylase.

The stabilities of coformycin, deoxycoformycin, and their 5′-phosphates, have been examined as a function of pH, and nature of the buffer medium. In particular, all exhibit instability in acid and neutral media, but are relatively stable in the vicinity of pH 9. Some biological aspects of the overall results are presented.

Introduction

Coformycin, (8*R*)-3-β-D-ribofuranosyl-3,6,7,8-tetrahydroimidazo-[4,5-*d*] [1,3]diazepin-8-ol, and 2′-deoxycoformycin (Scheme 1) are the most potent known inhibitors of adenosine deaminase [1, 2], with K_i values of the order of 10^{-10} M and 2.5×10^{-12} M, respectively (see ref. [3] for review). Both of these are classified as tight-binding inhibitors and, since they possess a tetrahedral carbon at the position corresponding to that in adenosine which undergoes deamination, are considered as transition state inhibitors of the enzyme [3, 4]. Deoxycoformycin is also a selective immunosuppressive agent, and is presently undergoing clinical trials in tumour chemotherapy [5].

The foregoing compounds also inhibit 5′-AMP deaminase, but with higher inhibition constants. Agarwal and Parks [2] report K_i values of 5×10^{-8} M for coformycin and 2×10^{-6} M for deoxycoformycin, with non-competitive inhibition, whereas Frieden *et al.* [6] found values of 2×10^{-8} M and 0.4×10^{-6} M, and claim competitive inhibition. However, when deoxycoformycin was converted to the 5′-phosphate, K_i decreased from about 10^{-6} M to about 10^{-9} M [7]. Since coformycin is a stronger inhibitor of AMP



Scheme 1. Coformycin (R = OH) and 2′-deoxycoformycin (R = H), with ring numbering system of the aglycon.

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deaminase than deoxycoformycin, it was suggested that coformycin-5′-phosphate might be an even more potent inhibitor of adenylate deaminase than 2′-deoxycoformycin-5′-phosphate.

The mode of action of deoxycoformycin has been generally considered to be related to its inhibition of adenosine deaminase. The finding that deoxycoformycin-5′-phosphate is a potent inhibitor of adenylate deaminase [6, 7], and that both coformycin and deoxycoformycin undergo intracellular phosphorylation [5, 8–10], with incorporation of deoxycoformycin into DNA [10], underline the potential significance of the phosphorylated forms. We have, therefore, prepared the 5′-phosphates of coformycin and deoxycoformycin and examined some of their properties, including their possible role in several enzymatic systems involved in nucleoside and nucleotide metabolism.

Materials

Coformycin and 2′-deoxycoformycin were gifts of Dr. V. L. Narayanan and the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, Md., USA).

Snake venom 5′-nucleotidase, calf intestinal mucosa adenosine deaminase, bovine spleen purine nucleoside phosphorylase, and rabbit muscle adenylate deaminase, were all purified preparations obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Wheat shoot phosphotransferase was prepared as elsewhere described [11, 12].

p-Nitrophenylphosphate, used as a donor in enzymatic phosphorylation, and *p*-nitrophenol were products of Merck (Darmstadt, GFR). Analytical grade Servachrom XAD-4 was a product of Serva (Heidelberg, GFR).

Methods

UV absorption spectra were followed with the aid of a Zeiss (Jena, GDR) Specord UV-VIS instrument, using 10-mm pathlength Spectrosil cuvettes.

^{1}H NMR spectra of coformycin and coformycin-5′-phosphate were recorded for solutions in ^2H_2O, with 2,2,3,3-tetradeutero-3(trimethylsilyl)-propionate as internal standard, on a Bruker 270 MHz spectrometer.

Thin layer chromatography was with Merck cellulose F_{254} plates, using two solvent systems: (A) isopropanol: water: 25% NH_4OH (4:4:1, v/v); (B) 1 M ammonium acetate: 96% ethanol (2:5, v/v).

Enzymatic activities were followed by sensitive fluorimetric methods, as described elsewhere, for 5′-nucleotidase [13], adenosine deaminase [14], purine nucleoside phosphorylase [15], and adenylate deaminase (in preparation).

Results

Enzymatic phosphorylation

In view of the lability of coformycin (see below), particularly pronounced in acid medium, attempts were first made to phosphorylate this compound enzymatically with the wheat shoot phosphotransferase system [11, 12], which is relatively non-specific with regard to the nature of the aglycon. Somewhat to our surprise, little or no phosphorylated product was obtained. Repeated trials eventually led to yields of 5% or less of coformycin-5′-phosphate, evaluated by TLC on cellulose plates with the solvent system B, which gave a good separation of coformycin-5′-phosphate (R_f = 0.07), coformycin (R_f = 0.58), *p*-nitrophenylphosphate, used as a phosphate donor (R_f = 0.33) and *p*-nitrophenol (R_f = 0.89). This constitutes a rather rare example of a nucleoside analogue relatively resistant to phosphorylation with wheat shoot phosphotransferase [11, 12]. It was initially thought that this might be due to the fact that the wheat shoot enzyme exhibits an optimum pH ~ 4.5, at which the aglycon of coformycin is largely protonated, pK ~ 5.4 [16] and undergoes time-dependent ring opening.

However, this cannot be the case since Frieden *et al.* [7] reported the enzymatic phosphorylation of 2′-deoxycoformycin in 14% yield with the phosphotransferase of *Serratia marcescens*, the pH optimum for which is 5.4, at which the aglycon is 50% protonated. With our system, elevation of the pH to 5 gave no improvement in the yield of coformycin-5′-phosphate. We then directed our attention to 2′-deoxycoformycin and found that, with a fresh preparation of the wheat shoot enzyme which phosphorylated cytidine (the aglycone of which is also 50% protonated at pH 4.5) in 80% yield, phosphorylation of deoxycoformycin proceeded readily to the extent of 15–20%. Under these conditions phosphorylation of coformycin was not improved, and remained at a level below 5%.

Chemical phosphorylation

Following a series of preliminary trials, coformycin was subjected to phosphorylation essentially accord-

ing to the procedure of Yoshikawa *et al.* [17], with some modifications. A suspension of 25 mg coformycin in 0.7 ml trimethylphosphate was stirred for 10 min at room temperature, cooled to −10 °C, followed by addition of 2 µl water, and 50 µl (0.54 mmol) $POCl_3$. Stirring was continued for an additional 30 min, during which the reaction mixture was brought to 0 °C, and the resulting clear solution stored in the cold room (4 °C) for 2–3 h. All subsequent steps (unless otherwise indicated) were performed in the cold room, and evaporations under reduced pressure at temperatures below 30 °C. To the foregoing reaction mixture was added an ice-cold solution of 300 mg $NaHCO_3$ in 4 ml water and the reaction mixture stirred vigorously at room temperature for 15 min to remove evolved CO_2. The mixture was then diluted with water to a total volume of about 20 ml, and deposited on a 2 × 25 cm column of DEAE Sephadex A-25 (HCO_3^-). The column was washed with water to remove unreacted coformycin (330 OD_{282} units), and elution then conducted with a linear gradient of 0–0.5 M triethylammonium bicarbonate, with collection of 18-ml fractions. The phosphorylated product eluted as a symmetrical peak at about 0.18 M (fractions 33–42). The pooled fractions were brought to dryness under reduced pressure, and again brought to dryness twice from water, and twice from aqueous ethanol, to remove triethylammonium carbonate.

The product, appreciably contaminated with inorganic phosphate, was next desalted on a 0.6 × 22 cm column of 100–200 µm Servachrom XAD-4. The column was first washed with methanol/isopropanol until the eluate exhibited an $OD_{222} < 0.1$, washed again with 250 ml 1 M triethylammonium bicarbonate, and then equilibrated with 0.05 M triethylammonium bicarbonate overnight. The phosphorylated product was taken up in 25 ml of the same solvent, deposited on the column, and eluted with the same solvent (with collection of 18-ml fractions) to give inorganic phosphate (fractions 1–4), followed by the nucleotide (fractions 7–23). The latter were pooled, brought to dryness, taken up in water and again brought to dryness (water pump), followed by four evaporations from aqueous ethanol (oil pump). The final residue was dissolved in water and lyophilized and dried over P_2O_5 to give 10 mg of the triethylammonium salt (20% yield with respect to initial coformycin).

The resulting coformycin-5′-phosphate exhibited a UV absorption spectrum identical with that for coformycin [16]. It was chromatographically homogeneous on cellulose F_{254} plates, with both solvent systems A (R_f = 0.49 as compared to 0.71 for coformycin) and B (R_f = 0.07 with R_f = 0.58 for coformycin).

Treatment of the coformycin-5′-phosphate with an excess of snake venom 5′-nucleotidase in 0.2 M Tris-HCl buffer pH 8.9 at 37 °C led to its slow conversion to coformycin, at about 2% the rate of dephosphorylation of 5′-AMP. The reaction was quantitative after 10 h. Under these conditions coformycin-5′-phosphate proved fully stable, as judged by chromatography, UV absorption and NMR spectroscopy. It should be recalled that 2′-deoxycoformycin-5′-phosphate is also dephosphorylated, very slowly, by snake venom 5′-nucleotidase [7].

^{1}H NMR spectroscopy

Apart from the foregoing criteria, the structure of the product was additionally, and unequivocally, confirmed by comparison of its ^{1}H NMR spectrum with the spectrum of the parent coformycin. The spectrum of the nucleoside was essentially identical with that reported by Hawkins *et al.* [18]. For the nucleotide, phosphorylation at the 5′-position was shown by the coupling constants J(5′,P) ~ 3.5 Hz and J(5″,P) ~ 5 Hz. Otherwise the spectra were very similar, with the exception that the chemical shifts of H(2) and H(5) in coformycin (see Scheme 1), 7.702 ppm and 7.202 ppm, respectively, were displaced to 8.179 ppm and 7.348 ppm in coformycin-5′-phosphate. If the assignments of H(2) and H(5) are indeed correct (*cf.* ref. [18]), this indicates that the nucleotide exhibits a *syn-anti* equilibrium about the glycosidic bond, predominantly in favour of the form *anti*, as shown in Scheme 1. These data should prove useful in studies on the conformations adopted by coformycin, and its 5′-phosphate, on binding to adenosine deaminase and adenylate deaminase, respectively. Detailed analyses of the ^{1}H, ^{13}C and ^{31}P NMR spectra of coformycin and coformycin-5′-phosphate are under way.

Lability of nucleosides and nucleotides

2′-Deoxycoformycin in aqueous solution is labile, particularly in acid medium [16], due to opening of the ring aglycon and, to a lesser extent, epimerization of the active *R* and inactive *S* forms [19], and

cleavage of the glycosidic bond (D. C. Baker, personal communication). Instability of deoxycoformycin and its phosphorylated forms has also been observed *in vivo* [5, 10].

We have examined susceptibility to ring opening, the major source of lability, and easily followed by the accompanying decrease of the UV absorption maximum at 282 nm. Lability of coformycin as a function of pH was found to be similar to that reported by Dion *et al.* [16] for deoxycoformycin. However, at pH ~ 9, both nucleosides were fully stable for 24 h at 37 °C. In acid medium, *e.g.* pH 4.5, in 1 M acetate buffer, coformycin underwent ~ 25% ring opening, but in 0.01 M acetate only 9%. This effect was not due to a difference in ionic strength, but to buffer concentration. The nature of the buffer, particularly at neutral pH, also affected the rate of ring opening.

In acid and neutral media, the 5′-phosphates of both nucleosides were more unstable than the nucleosides, *e.g.* at pH 7, coformycin-5′-phosphate was 10-fold more labile than coformycin.

The most striking observation was that at pH ~ 9 the 5′-phosphates of both coformycin and deoxycoformycin were fully stable for 24 h at 37 °C. This stability in alkaline medium of both the nucleosides and nucleotides should prove useful in developing methods of extraction from cellular material for subsequent analyses.

Finally, and in contrast to deoxycoformycin, ^{1}H NMR spectroscopy demonstrated that neither coformycin, nor its 5′-phosphate, underwent cleavage of the glycosidic bond.

Properties in some enzyme systems

In view of the increasing evidence that the mode of action of deoxycoformycin is not limited to its inhibition of adenosine deaminase, it appeared of value to examine the behaviour of coformycin and deoxycoformycin, and their 5′-phosphates, in several enzyme systems, as follows:

Snake venom 5′-nucleotidase. The poor substrate properties of coformycin-5′-phosphate and deoxycoformycin-5′-phosphate in this enzyme system raised the question as to whether they may not be inhibitors. Using a procedure elsewhere described [13] for kinetic studies on 5′-nucleotidase, neither of these compounds was found to sensibly inhibit the enzyme. Estimated inhibition constants for both compounds gave values of $K_i > 0.3$ mM. It will be of interest to examine the substrate and inhibitor properties of these compounds with 5′-nucleotidases of mammalian origin, some of which exhibit specificities differing from those of the snake venom enzyme [13].

Purine nucleoside phosphorylase. It was previously reported that 2′-deoxycoformycin does not significantly inhibit purine nucleoside phosphorylase from human leukemic granulocytes [20]. Using a purified enzyme from bovine spleen, and a fluorimetric procedure for kinetic studies, with 7-methyl-guanosine as substrate [15], it was found that neither coformycin nor deoxycoformycin was a significant inhibitor, the estimated values of K_i for each being > 0.5 mM. It may be concluded that both are not, or are very poor, substrates for the mammalian enzyme. However, this may not apply to the bacterial enzyme, for which adenosine is a substrate.

Adenosine deaminase. Since coformycin and deoxycoformycin are potent inhibitors of adenosine deaminase, and also good inhibitors of adenylate deaminase [2, 3], it is pertinent to ask whether the nucleotides exhibit inhibitory properties *vs.* adenosine deaminase. Using a purified preparation of the enzyme from calf intestinal mucosa, and a sensitive fluorimetric procedure for adenosine deaminase activity [14], neither of the nucleotides was found to exhibit significant inhibition. Kinetic studies, using procedures described elsewhere [14], indicated K_i values for both nucleotides well above 0.2 mM, as compared to 10^{-10} M and 2.5×10^{-12} M for coformycin and 2′-deoxycoformycin, respectively [3].

Adenylate deaminase. The enzyme was a purified preparation (Sigma) from rabbit muscle, and the substrate was formycin-5′-phosphate, a fluorescent analogue of 5′-AMP. Enzyme activity was followed by the accompanying decrease in fluorescence (in preparation) as elsewhere described for adenosine deaminase activity [14]. With the aid of the kinetic method described by Frieden *et al.* [7] for following inhibition by deoxycoformycin-5′-phosphate, it was confirmed that both coformycin-5′-phosphate and deoxycoformycin-5′-phosphate were equally potent inhibitors. For coformycin-5′-phosphate the rate constant for association with the enzyme was $k_{on} = 0.9 \times 10^5$ M^{-1}s^{-1}, in agreement with Frieden *et al.* [6]. For both nucleotides, $K_i \leq 10^{-9}$ M. However, because of the known lability of 5′-AMP deaminase, it

could not be established unequivocally whether K_i for coformycin-5′-phosphate was indeed less than that for deoxycoformycin-5′-phosphate, as suggested [7], but not shown experimentally [6]. This is being further investigated.

Discussion

The ability of 2′-deoxycoformycin and, to a lesser extent, of coformycin to undergo phosphorylation by phosphotransferases is relevant to the metabolism and mechanisms of action of these nucleoside analogues in mammalian cells. In L1210 cells, deoxycoformycin is converted to the 5′-phosphate to a level of about 15% [9]. Coformycin is metabolized in L5178Y cells to the 5′-mono-, di- and triphosphates to the extent of 50% [8]. In human T lymphoblastoid cells, deoxycoformycin is phosphorylated stepwise to the triphosphate, which is incorporated into DNA [10]. In the latter study, the use of mutant cell lines deficient in enzyme activities indicated that initial phosphorylation of deoxycoformycin was not due to adenosine kinase or deoxycytidine kinase [10]. The failure of adenosine kinase to phosphorylate deoxycoformycin is understandable, since this enzyme very poorly phosphorylates deoxyadenosine [21], and deoxycoformycin is not a substrate or inhibitor for rabbit liver adenosine kinase [22]; it would be of interest to examine whether coformycin is. Deoxycytidine kinase does phosphorylate deoxyadenosine, and the failure of this enzyme to phosphorylate deoxycoformycin points to possible involvement of another enzyme. It has, in fact, been reported recently [23] that mammalian cells exhibit phosphotransferase activity which phosphorylates ribo- and deoxyribonucleosides; and Siaw and Coleman [10] have also drawn attention to such activity.

It should also be noted that protozoan parasites, require purine salvage pathways for survival. Among the *Leishmania* spp, one such unique very active enzyme is a purine nucleoside phosphotransferase, which transfers phosphate from a variety of donors to the 5′ of purine nucleosides [24]. Nucleoside analogues, *e.g.* the riboside of allopurinol or of formycin B, are phosphorylated in these parasites, and then act as inhibitors of other essential enzymes in purine metabolism. It would be of considerable interest to examine the behaviour of coformycin and deoxycoformycin in such parasites.

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