STUDIES ON INHIBITION OF ADENOSINE DEAMINASE BY ISOCOFORMYCIN *IN VITRO* AND *IN VIVO*

MASAMI SHIMAZAKI, YOSHIKI KUMADA, TOMIO TAKEUCHI, HAMAO UMEZAWA
Institute of Microbial Chemistry,
Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

and KIYOSHI WATANABE
Biochemical Research Laboratories, Kanegafuchi Chemical Industry Co., Ltd.,
Takasago, Hyogo 676, Japan

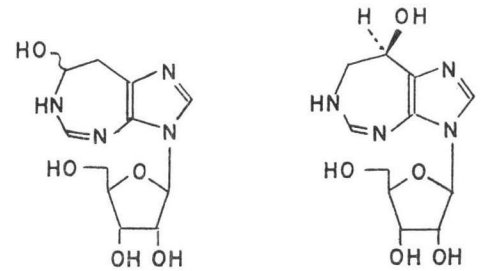
(Received for publication March 19, 1979)

Isocoformycin is a structural isomer of coformycin which has been demonstrated to be a potent inhibitor of adenosine deaminase. Isocoformycin showed a weaker inhibition of this enzyme than coformycin; the binding of coformycin to enzyme was irreversible, but isocoformycin inhibition was competitive with substrate. The *Ki* value of isocoformycin was $4.5 \sim 10 \times 10^{-8}$ M. Following intraperitoneal injection of isocoformycin in mice, the adenosine deaminase activity of homogenates of several organs was determined and the following ED_{50} values (50% inhibition doses) were observed: 29 mg/kg for thymus, 13 mg/kg for spleen, 80 mg/kg for liver and 20 mg/kg for kidney. The inhibition of adenosine deaminase in rabbit blood *in vitro* was also tested in comparison with coformycin.

UMEZAWA *et al.*[1,2] reported that coformycin is a strong inhibitor of adenosine deaminase (EC 3,5,4,4) and exhibits strong synergistic effects with formycin A and arabinosyl adenine in inhibition of the growth of bacteria, EHRLICH carcinoma cells and YOSHIDA rat sarcoma cells. Moreover, coformycin (Fig. 1) had a unique structure[3] as an inhibitor of adenosine deaminase. 2′-Deoxycoformycin[4] also exhibits a strong inhibition of the enzyme and strong synergistic effects with adenosine analogs[5~9]. These inhibitors bind tightly to adenosine deaminase[10]. The effect or toxicity of such strongly bound inhibitors may persist for longer periods than that of inhibitors which inhibit the enzyme competitively. We have described the synthesis of isocoformycin (3-β-D-ribofuranosyl-3,6,7,8-tetrahydroimidazo [4,5-d][1,3]diazepin-7-ol, Fig. 1)[11]. This compound was found to be a competitive inhibitor of adenosine deaminase.

In this paper, studies on the inhibition of adenosine deaminase by isocoformycin *in vitro* and *in vivo* are reported.

Fig. 1. The structures of isocoformycin and coformycin.



Isocoformycin Coformycin

Materials and Methods

Formycin A and coformycin were supplied by Meiji Seika Co. Adenosine was obtained from a commercial source and purified by recrystallization from water. 9-β-D-Arabinofuranosyladenine (ara-A) was obtained from Drug Research and Development, National Cancer Institute, Bethesda, U.S.A. Adenosine deaminase (Type I, 230 units/mg protein) of calf intestinal mucosa was purchased from

Sigma Chemical Co. 9-β-D-Arabinofuranosylhypoxanthine (ara-H) was synthesized from ara-A by oxidation with sodium nitrite.

Kinetic Studies

ACKERMANN-POTTER analysis was carried out by the method described by CHA *et al.*[10] Varied amounts of adenosine deaminase were incubated in a total volume of 1.9 ml of 50 mM sodium phosphate buffer, pH 7.5, containing isocoformycin (or coformycin) at varied concentrations. After incubation for 50 minutes at room temperature, 0.1 ml of 2 mM adenosine was added and the reaction was followed by measuring the decrease in absorbancy at 265 nm at room temperature. Alternatively, the enzyme reaction was carried out without preincubation with isocoformycin and the results examined by a LINEWEAVER-BURK plot. When ara-A or formycin A was used as the substrate, the decrease in the optical density at 259 nm or 295 nm, respectively was recorded.

Inhibition of Adenosine Deaminase by Isocoformycin *in vivo*

Isocoformycin dissolved in 0.9% NaCl solution was injected intraperitoneally to *ddY* mice at 100 mg/kg, 25 mg/kg and 6.25 mg/kg. For each dose, 3 mice were used. One hour after the injection, mice were sacrificed and thymus, spleen, liver and kidney were frozen immediately and weighed. They were homogenized in 2 volumes of 50 mM cold Tris-HCl buffer (pH 7.5) with a motor-driven glass pestle. Fifty μl of each homogenate were added to 450 μl of a reaction mixture containing 50 μl of 0.5 M Tris-HCl buffer (pH 7.5) and 50 μl of 10 mM [^{3}H]-ara-A (0.3 μci/assay) at 0°C. The mixture was incubated for 5, 10, 20 and 30 minutes at 37°C. The reaction was stopped by heating in a boiling water bath for 3 minutes and 100 μl aliquots were subjected to high-voltage paper electrophoresis (3,500 V, 20 minutes) using Toyo Filter Paper No. 51. By this method, a complete separation of ara-H from ara-A was obtained: Rm of ara-A, 0.70~0.73; Rm of ara-H, 0.20~0.23; Rm is the mobility of a sample relative to alanine as standard. The area of ara-H was cut out and determined by liquid scintillation counting. From the results, the doses which caused the reduction of adenosine deaminase activities of thymus, spleen, liver and kidney to 50% of the control were obtained.

Inhibition of Adenosine Deaminase in Rabbit Blood by Isocoformycin or Coformycin *in vitro*

One ml of an aqueous solution of isocoformycin (or coformycin) at varied concentrations was added to 1 ml of formycin A solution (326 μg/ml) or 1 ml of ara-A solution (336 μg/ml). The reaction was started by addition of 1.0 ml of rabbit blood (10 ml of rabbit blood was taken into a syringe containing 100 units heparin) and the mixture incubated at 37°C for 10 minutes. The reaction was stopped by addition of 3.0 ml of methanol and the precipitate removed by centrifugation; 6 μl of the supernatant were subjected to high pressure liquid chromatography (JASCO FLC-A10): solid phase, JASCO CV-01-500 (2.3 mm × 500 mm); mobile phase, 0.01 M KH_2PO_4 of pH 6.0 for the analysis of formycin or at pH 4.7 for the analysis of ara-A; flow rate, 0.5 ml/min; column pressure, 100 kg/cm^2; column temperature, ambient; detecter, UVIDEC-100 (wave length 280 nm); chart speed, 0.5 cm/min.

Result

Kinetic Studies

Coformycin has been reported by CHA *et al.*[10] to be a tight-binding inhibitor of adenosine deaminase. The binding of isocoformycin was examined by ACKERMANN-POTTER analysis. As shown in Fig. 2, coformycin was a stoichiometric inhibitor, but isocoformycin was not a tight-binding inhibitor. The inhibition by isocoformycin was competitive with the substrate adenosine, as illustrated in Fig. 3. The *Ki* value for isocoformycin was obtained by a LINEWEAVER-BURK plot of the results. Table 1 indicates the *Ki* values of isocoformycin and coformycin when adenosine, ara-A or formycin A were used as the substrate.

Inhibition of Adenosine Deaminase by Isocoformycin *In vivo*

Fig. 4 indicates the adenosine deaminase activity of thymus, spleen, liver and kidney of mice

Fig. 2. ACKERMANN-POTTER plot of adenosine deaminase.
Preincubated with coformycin (A) and isocoformycin (B) (Substrate: adenosine)

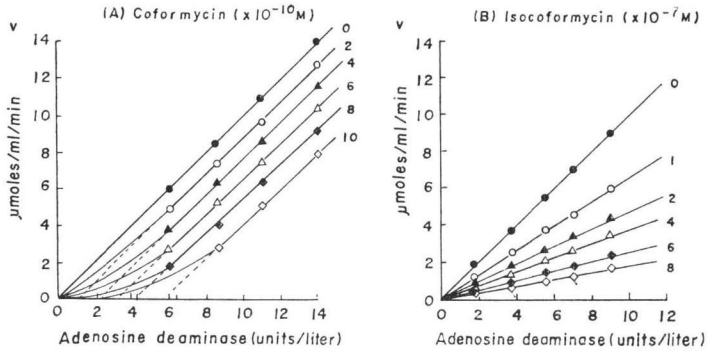
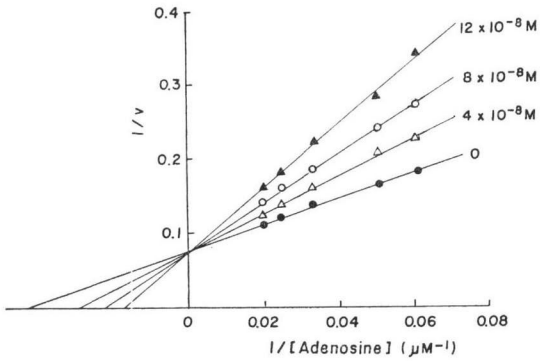


Table 1. The *Ki* values of isocoformycin and coformycin against adenosine deaminase.

Inhibitor	Substrates		
	Formycin A ($Km=3.0\times10^{-4}$ M)	Adenosine ($Km=2.2\times10^{-5}$ M)	ara-A ($Km=1.0\times10^{-4}$ M)
Isocoformycin	4.5×10^{-8} M (competitive)	8.0×10^{-8} M (competitive)	1.0×10^{-7} M (competitive)
Coformycin	1.5×10^{-10} M	1.8×10^{-10} M	1.5×10^{-10} M

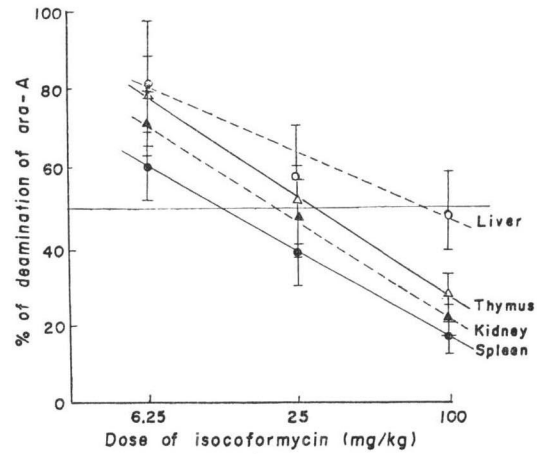
Fig. 3. Inhibition of adenosine deaminase by isocoformycin.



injected with isocoformycin relative to the control. From the curves in this figure, doses which reduced the activity of thymus, spleen, liver and kidney to 50% of the control were estimated to be 29 mg/kg, 13 mg/kg, 80 mg/kg and 20 mg/kg, respectively.

Fig. 4. Inhibition of deamination of ara-A by isocoformycin *in vivo*.

Values given are percentage of the control and represent averages of analyses on 3 mice. Each point indicates the mean value±S.D. of the percent deamination.

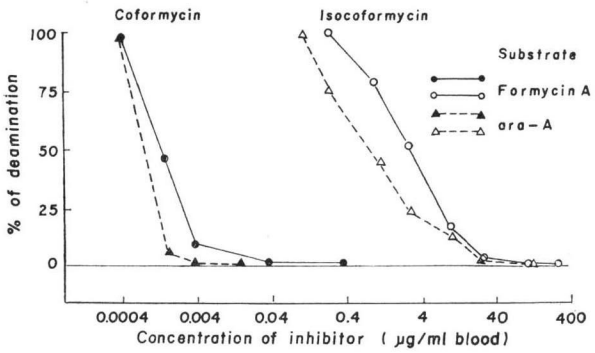


Inhibition of Adenosine Deaminase in Rabbit Blood by Isocoformycin and Coformycin *In vitro*

Inhibition of deamination of formycin A and ara-A in blood by isocoformycin and coformycin *in vitro* was examined. The results are shown in Fig. 5. When the ID_{50} value (50% inhibition con-

centration) of isocoformycin are compared with those of coformycin, the former is about 1,000~2,000 times weaker than the latter indicated as follows: 4 μg/ml or 1.5 μg/ml of isocoformycin required for 50% inhibition of the deamination of formycin A or ara-A respectively, compared to 0.0019 μg/ml or 0.0013 μg/ml of coformycin.

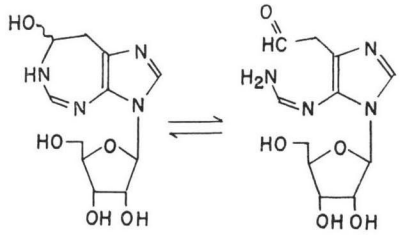
Fig. 5. Inhibition of adenosine deaminase of rabbit blood by coformycin and isocoformycin.



Discussion

As shown in Fig. 6, isocoformycin can have two stereoisomers due to changes in the stereochemistry of the hydroxyl group-binding carbon atom in the seven membered ring. It is thought that one of these epimers can be converted to the other through the intermediate ring-opened aldehyde form and both epimers exist in the aqueous solution. Coformycin does not possess these epimeric structures and is structurally more related to adenosine, ara-A and formycin than isocoformycin. These structure relationships imply that coformycin inhibits adenosine deaminase much more strongly than isocoformycin. As shown by the extremely small *Ki* value the binding of coformycin to the enzyme is apparently irreversible.

Fig. 6. Isocoformycin structures in solutions in equilibrium.



Although the activity of isocoformycin is much weaker than that of coformycin, the *Ki* value for adenosine deaminase was $4.5 \sim 10.0 \times 10^{-8}$ M and it is still a strong inhibitor of the enzyme. A small difference in *Ki* values was observed depending on substrates such as formycin, adenosine or ara-A. The *Ki* value should be independent of substrate and the small differences described previously may be due to experimental error.

Both coformycin[2,3] and isocoformycin exhibit inhibition of adenosine deaminase *in vivo*. The administration of 10~100 mg/kg decreased the deamination of ara-A in various organs to 50% of the control. As will be reported subsequently, isocoformycin has a much lower toxicity than coformycin.

References

1) Sawa, T.; Y. Fukagawa, I. Homma, T. Takeuchi & H. Umezawa: Mode of inhibition of coformycin on adenosine deaminase. J. Antibiotics, Ser. A 20: 227~231, 1967
2) Umezawa, H.; T. Sawa, Y. Fukagawa, I. Homma, M. Ishizuka & T. Takeuchi: Studies on formycin and formycin B in cells of Ehrlich carcinoma and *E. coli*. J. Antibiotics, Ser. A 20: 308~316, 1967
3) Nakamura, H.; G. Koyama, Y. Iitaka, M. Ohno, N. Yagisawa, S. Kondo, K. Maeda & H. Umezawa: Structure of coformycin and an unusual nucleoside of microbial origin. J. Am. Chem. Soc. 96: 4327, 1974
4) Woo, P. W. K.; H. W. Dion, S. M. Lange, L. F. Dahl & L. J. Durham: A novel adenosine and ara-A deaminase inhibitor, (R)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-d][1, 3]-diazepin-8-ol. J. Heterocyclic Chem. 11: 641~643, 1974
5) Plunkett, W. & S. S. Cohen: Two approaches that increase the activity of analogs of adenosine nucleoside in animal cells. Cancer Res. 35: 1547~1554, 1975
6) Cass, C. E. & T. H. Au-Yeung: Enhancement of 9-β-D-arabinofuranosyladenine cytotoxicity to mouse leukemia *in vitro* by 2'-deoxycoformycin. Cancer Res. 36: 1486~1491, 1976

7) LEPAGE, G. A.; L. S. WORTH & A. P. KIMBALL: Enhancement of the antitumor activity of arabinofuranosyladenine by 2'-deoxycoformycin. Cancer Res. 36: 1481~1485, 1976
8) CARON, N.; S. H. LEE & A. P. KIMBALL: Effects of 2'-deoxycoformycin, 9-β-D-arabinofuranosyladenine 5'-phosphate, and 1-β-D-arabinofuranosylcytosine triple combination therapy on intracerebral leukemia 1210. Cancer Res. 37: 3274~3279, 1977
9) ADAMSON, R. H.; D. W. ZAHAREVITZ & D. G. JONES: Enhancement of the biological activity of adenosine analogs by the adenosine deaminase inhibitor 2'-deoxycoformycin. Pharmacology 15: 84~89, 1977
10) CHA, S.; R. P. AGARWAL & R. E. PARKS, Jr.: Tight-binding inhibitors. II. Non-steady state nature of inhibition of milk xanthine oxidase by allopurinol and alloxanthine and of human erythrocytic adenosine deaminase by coformycin. Biochem. Pharmacol. 24: 2187~2197, 1975
11) SHIMAZAKI, M.; S. KONDO, K. MAEDA, M. OHNO & H. UMEZAWA: Synthesis of isocoformycin, an adenosine deaminase inhibitor of synthetic origin. J. Antibiotics 32: 537~538, 1979