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ENZYME INHIBITION TITRATION ASSAY FOR 2′-DEOXYCOFORMYCIN AND ITS APPLICATION TO THE STUDY OF THE RELATIONSHIP BETWEEN DRUG CONCENTRATION AND TISSUE ADENOSINE DEAMINASE IN DOGS AND RATS

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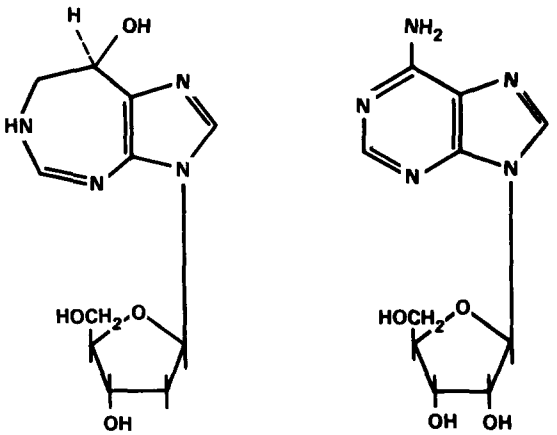
Abstract—An assay has been developed for 2′-deoxycoformycin (2′-dCF), utilizing the inhibition by the drug of the enzyme adenosine deaminase. This assay is suitable for the detection of 2′-dCF in tissue extracts and biological fluids with a maximal sensitivity of 2 nM. The assay has been used to study the disposition of the drug in foxhounds and to examine the relationship between tissue drug levels and residual tissue adenosine deaminase activity. 2′-dCF was administered intravenously to foxhounds at doses of 0.1, 0.25 and 1.0 mg/kg. The drug disappeared from plasma with α- and β-half times of 12–15 min and 90–120 min respectively. It was cleared into urine at a rate of 2.24 to 3.12 ml/min/kg. Of the administered drug, 54–83 per cent was recovered in the urine and another 10–15 per cent in the tissues at the time of death. Drug accumulation in tissues correlated with the adenosine deaminase content of the tissues. Residual adenosine deaminase activity was under 50 per cent of control activity in all tissues examined after 0.1 mg/kg of 2′-dCF and under 20 per cent of control activity in all tissues after 1.0 mg/kg of 2′-dCF. Rats given 2′-dCF showed initial inhibition of adenosine deaminase with recovery over time in tissues capable of new enzyme synthesis. The variable rates of recovery from enzyme inhibition may be determinants of tissue specific toxicity of 2′-dCF alone or 2′-dCF in combination with adenosine analogs. Dosage schedules are suggested for different clinical objectives.

2′-Deoxycoformycin (2′-dCF)(R-3-(2-deoxy-β-D-erythropentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol; covidaribine; pentostatin) (Fig. 1), a bacterial fermentation product of *Streptomyces antibioticus* isolated and characterized by Woo *et al.* [1], is a potent inhibitor of the enzyme adenosine deaminase (EC 3.5.4.4). We and others have shown previously that 2′-dCF potentiates the antitumor activity of adenosine analogs against murine leukemic cells *in vivo* and *in vitro* [2–5]. We have also demonstrated that the drug has immunosuppressive activity as measured by inhibition of delayed hypersensitivity skin response, allograft rejection, and lymphocyte responsiveness to mitogen stimulation *in vitro* [5–7]. In view of the potential clinical utility of this drug for cancer chemotherapy in combination with adenosine analogs and as an immunosuppressive agent if used alone, we have developed an enzyme inhibition titration method for assaying the drug in tissue extracts and biological fluids and have utilized this method to study some aspects of the disposition of the drug in dogs and rats, and also to explore the relationship between tissue levels of the drug and of its target enzyme adenosine deaminase. A preliminary account of these studies has appeared [8].



2′ – Deoxycoformycin **Adenosine**

Fig. 1. Structures of 2′-deoxycoformycin and adenosine.

MATERIALS AND METHODS

Materials. 2′-dCF (NSC 218321) and formycin A (7-amino-3-(β-D-ribofuranosyl)-pyrazolo[4,3-*d*]pyrimidine) (NSC 102811) were obtained from Dr. John Douros of the Developmental Therapeutics Program, National Cancer Institute. Calf intestinal adenosine deaminase [(type I) in 3.2 M $(NH_4)_2SO_4$] (sp. act. 255 units/mg of protein) and adenosine were obtained from the Sigma Chemical Co. (St. Louis, MO). Heparin was obtained from the Upjohn Co. (Kalamazoo, MI) and pentobarbital from Veterinary Labs (Lenexa, KA).

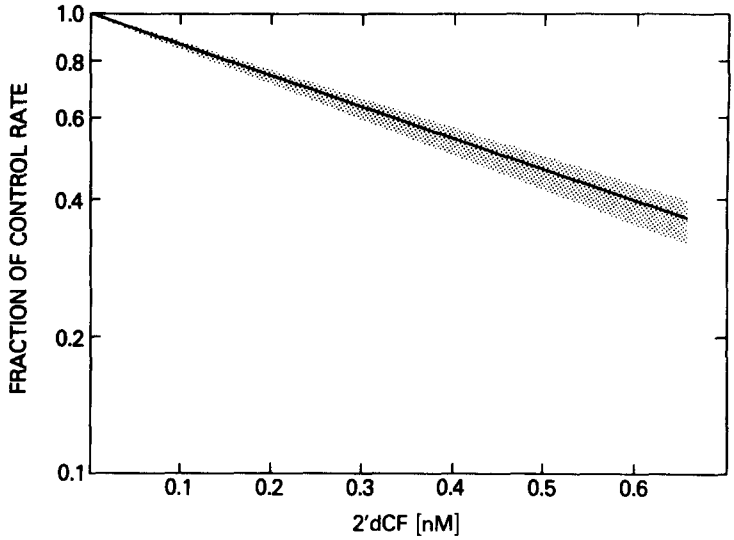


Fig. 2. Standard curve for determination of 2′-deoxycoformycin. Mean ± S.E.M. for the line was determined from the data of twelve separate determinations of the relationship between 2′-dCF cuvette concentration and per cent of control reaction rate. [Concentration [nM] = −1.50 log (fraction of control rate)].

2′-dCF assay. 2′-dCF was determined by the inhibition of the adenosine deaminase catalyzed conversion of adenosine to inosine ($\Delta\varepsilon = 8600\ M^{-1}\ cm^{-1}$) followed at 265 nm by means of a Gilford multiple sample absorbance recorder. Calf intestinal adenosine deaminase, 0.0067 units, and known quantities of 2′-dCF in the range of 0.2 to 1.0 pmoles (final concentration range, 0.2 to 1.0 nM in the cuvette) were incubated at 37° in 0.05 M potassium phosphate buffer, pH 7.4, in a 1-ml cuvette for 10 min. The reaction was started by the addition of adenosine to a concentration of 0.1 mM in the cuvette. In this way, twelve separate determinations of a standard curve were obtained. Figure 2 represents a plot of the standard curve obtained by averaging the lines obtained from twelve separate standard curve determinations ($r = 0.986$). This plot was used to determine all unknowns. Unknowns were determined by adjusting the cuvette concentration of the sample to obtain a rate of 50–85 per cent of the uninhibited control, representing a 2′-dCF concentration of 0.15 to 0.5 nM. Validation of the assay was carried out by adding to plasma and urine known concentrations of 2′-dCF (recovery equal to 98 ± 8 per cent). For determination of 2′-dCF in plasma, samples were diluted to appropriate cuvette concentrations and determined directly; for dilutions of less than 1:100, the plasma was boiled and the 100,000 *g* supernatant fraction was then diluted for use as the sample, as some inhibition of deamination by concentrated drug-free plasma was detectable. Barondy *et al.* [9] have shown that 2′-dCF binds to protein. However, the dissociation constant is sufficiently high (2×10^{-3} M) that free drug approximates total drug at the concentrations dealt with in this study. Cerebrospinal fluid (CSF) and urine samples

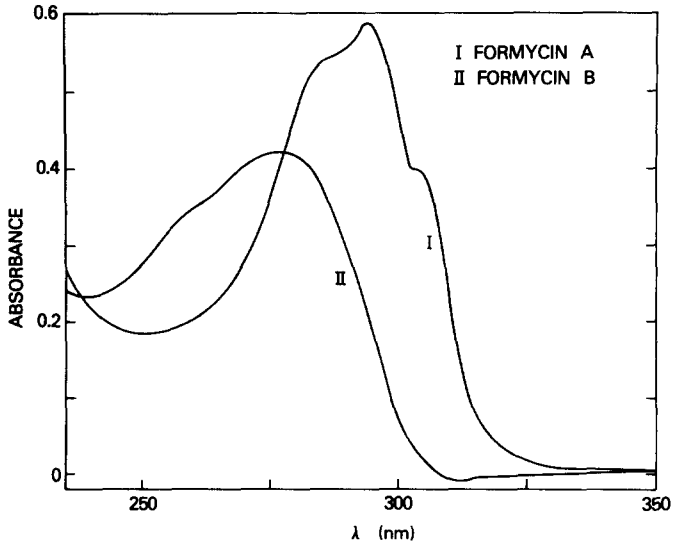


Fig. 3. Absorption spectra of formycin A and formycin B (0.1 mM).

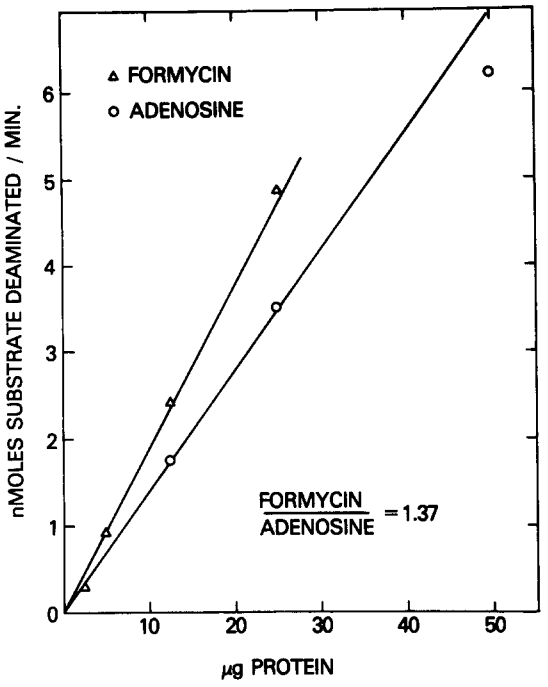


Fig. 4. Relationship of reaction velocity and adenosine deaminase content for the deamination of formycin A and adenosine. Varying amounts of calf intestinal adenosine deaminase were placed in a 1-ml cuvette with 0.05 M potassium phosphate buffer, pH 7.4, and either 0.1 mM adenosine or 0.1 mM formycin A. The reaction with adenosine was followed on a Gilford multiple sample absorbance recorder at 265 nm and that with formycin was followed at 305 nm.

were diluted to appropriate cuvette concentration and determined directly. No inhibitory activity was encountered with drug-free CSF at concentrations under 1:40 in the cuvette. The 2′-dCF concentration was always determined at urinary dilutions which yielded no endogenous inhibition. For tissue 2′-dCF determination, 2-g samples of tissues were homogenized in 2 vol. of Tris–HCl buffer, pH 8.6, with a Brinkmann Polytron high speed homogenizer. Homogenates were boiled for 5 min and the 100,000 *g* supernatant fraction was used as the sample.

Adenosine deaminase assay. Adenosine deaminase activity was measured spectrophotometrically in supernatant fractions from homogenized tissue samples on a Gilford multiple sample absorbance recorder. Since the soluble proteins in the tissue extract absorb strongly at 265 nm, the amount of extract that can be added to the cuvette for assay at this wavelength, with adenosine as substrate, is small and, hence, sensitivity is poor. In order to assay adenosine deaminase activity at a higher wavelength, we chose instead to use formycin A as substrate. Adenosine deaminase catalyzes the deamination of formycin A to formycin B with a K_m of 2 mM and a V_{max} of 3 μmoles/min/mg of protein. The change in molar extinction coefficient at 305 nm during the reaction is 6000 M^{-1} cm^{-1} (Fig. 3) [4, 10]. The sample and 0.05 M potassium phosphate buffer, pH 7.4, were added to a 1-ml cuvette at 37°; the reaction was started by the addition of formycin A at a final concentration of 0.1 mM and followed by measuring the rate of decrease in absorbance at 305 nm. Using calf intestinal adenosine deaminase, under the conditions described, the reaction rate is linear with adenosine deaminase concentration and slightly faster than the rate observed with adenosine as substrate (Fig. 4). Tissue samples were homogenized in 2 vol. of 0.05 M potassium phosphate buffer, pH 7.4, in a Brinkmann Polytron, centrifuged at 100,000 *g* × 30 min at 4°, and the supernatant fractions were used for the determination of enzyme activity on the same day. Protein determinations on the tissue extracts were performed by the method of Lowry *et al.* [11].

Dog studies. Adult female foxhounds (14–17 kg) from the NIH Animal Facility were used for these studies. Doses of 2′-dCF were prepared at a concentration of 1 mg/ml in phosphate buffered normal saline, pH 7.4. The animals were anesthetized with pentobarbital, 30 mg/kg i.v., the femoral artery and vein were cannulated, a needle was placed in the cisterna magna by suboccipital puncture, and a Foley catheter was inserted into the urinary bladder. The drug was administered as a bolus into the femoral vein; all samples for plasma determination were taken from the femoral artery into a heparinized syringe, kept cold on ice, and centrifuged for 10 min at 1200 *g*. The plasma was separated from the red cells and stored at 4°. Cerebrospinal fluid and urine samples were likewise cooled on ice and stored at 4°. The animals were kept anesthetized for a 6-hr period while multiple plasma, CSF, and urine samples were obtained. All indwelling catheters were then removed, and the animals allowed to regain consciousness and held with free access to food and water in metabolic cages for 24–48 hr; they were then killed by a lethal intravenous dose of pentobarbital. Various tissues were removed, trimmed and weighed; 2-g samples were frozen and stored at −20° for later determination of tissue 2′-dCF and adenosine deaminase. Two dogs each received 2′-dCF intravenously at 0.1 mg/kg, 0.25 mg/kg and 1.0 mg/kg. One dog received 0.25 mg/kg, intraperitoneally. The doses were in the same range as those doses found to be effective therapeutically in our previous studies of the combination chemotherapy of murine leukemia. All the results presented for either 2′-dCF or adenosine deaminase are the means of triplicate determinations on samples from each of the two dogs treated at each dose level.

Rat studies. Adult, female Sprague–Dawley rats (200–250 g) were given 2′-dCF, i.p., at a dose level of 0.25 mg/kg. Two animals each were killed at 0, 1, 4, 8, 16 and 24 hr. Two-g samples of red cells, jejunum, spleen and thymus were frozen and stored at −20° for later determination of tissue adenosine deaminase activity.

RESULTS

Plasma and CSF pharmacokinetics. The plasma disappearance curves after i.v. bolus administration of 2′-dCF at the three dose levels are shown in Fig. 5. Initial plasma concentrations are 1.45, 2.9 and 12.75 mM respectively. Plasma concentrations remained above 1 mM for 15, 60 and 180 min respectively. The curves demonstrate a rapid distribution $T_\frac{1}{2}$ of 12–15 min and a β-$T_\frac{1}{2}$ of 90–120 min. Extrapolating the β line to t_0 gives an estimate of drug distribution volume of 60–70 per cent of total body mass, approximating total body water.

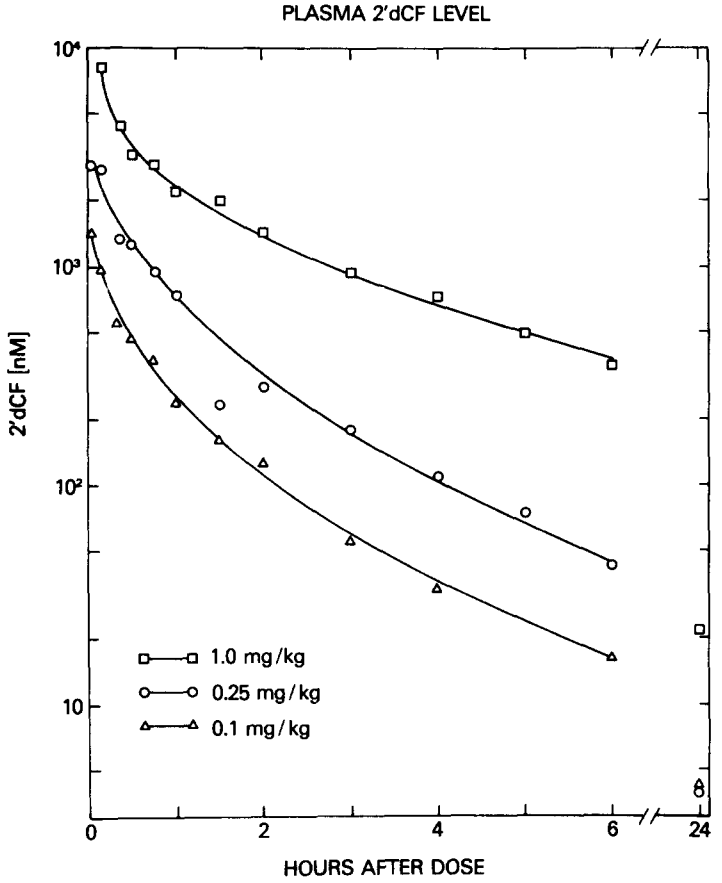


Fig. 5. Plasma levels of 2′-dCF in dogs. Values represent the mean of triplicate determinations from two dogs given each dose.

The CSF 2′-dCF levels of the three doses are shown in Fig. 6. Peak CSF concentrations are reached 2–3 hr after injection and they are about 10 per cent of the simultaneous plasma level. CSF and plasma 2′-dCF levels were equal at 24 hr.

Excretion. Only urinary excretion of the drug was determined. Biliary concentrations of drug could not be obtained because of inhibition of the enzymatic reaction by drug-free bile. Fifty-four to 83 per cent of the administered drug was recovered in the urine, 58–96 per cent of this within the first 6 hr. A further 10–15 per cent of the administered drug was recovered from the tissues so that we were able to account for 72–96 per cent of administered drug in all cases. The estimated clearance of drug in the urine was 2.24 to 3.12 ml/min/kg, equal to the inulin clearance for dogs.

Tissue distribution of 2′-dCF. Tissue distribution of 2′-dCF after administration of two dose levels is shown in Fig. 7. The drug is concentrated in all tissues relative to plasma at 24 hr after 0.1 mg/kg or at 48 hr after 1.0 mg/kg, and reaches the highest levels in tissues which are richest in adenosine deaminase. While lung tissue is the richest source of adenosine deaminase in dogs, the accumulation demonstrated here at the highest dose is not strictly proportional to the adenosine deaminase level. To investigate whether this accumulation of drug in lung is an effect of first passage of an intravenously administered bolus through that organ, one dog was given 0.25 mg/kg of 2′-dCF by intraperitoneal injection (data not shown). The distribution of

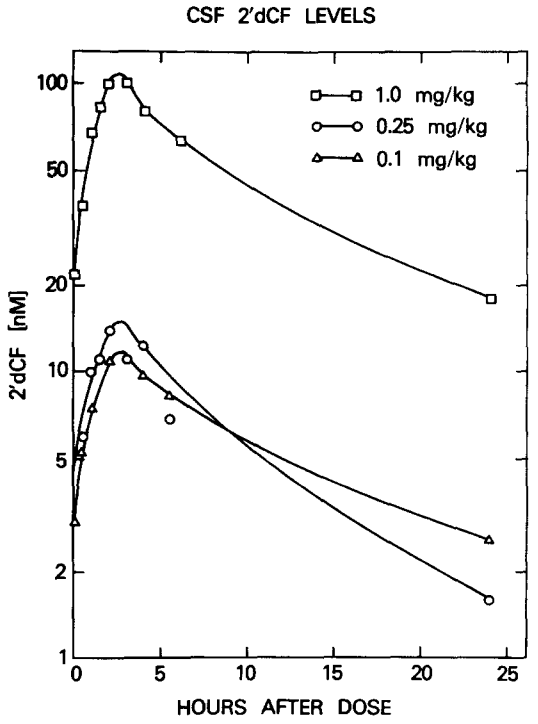


Fig. 6. CSF levels of 2′-dCF in dogs. Values represent the mean of triplicate determinations from two dogs given each dose.

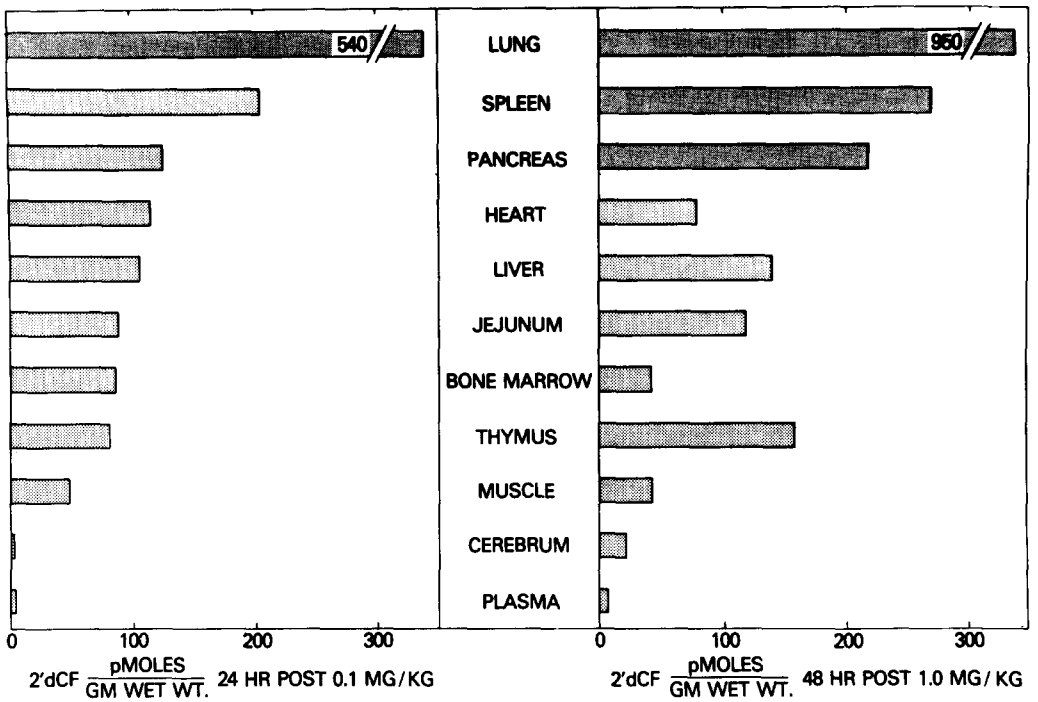


Fig. 7. Tissue distribution of 2′-dCF 24 hr after 1.0 mg/kg i.v. and 48 hr after 1.0 mg/kg i.v. Values are the mean of triplicate determinations from two dogs given each dose.

drug in its tissues was not significantly different from that in the dogs to which the same dose had been administered intravenously.

Tissue adenosine deaminase. Adenosine deaminase is an enzyme which is distributed widely in all tissues of the body. Residual tissue adenosine deaminase activity at the time of death after 2′-dCF injection, as compared to the uninhibited activity determined in several tissues of two control dogs, is shown in Fig. 8. All tissues examined had less than 20 per cent of the control enzyme activity at 48 hr after 1 mg/kg of 2′-dCF. Even at 0.1 mg/kg all tissues except jejunum and thymus had less than 20 per cent of the control activity. Reduction of activity to less than 10 per cent of the control occurred in liver, heart, kidney and muscle at this low dose. At 0.25 mg/kg this degree of reduction in ADA activity occurred in the pancreas, spleen, lung, liver, heart, kidney and muscle. In most cases the greatest

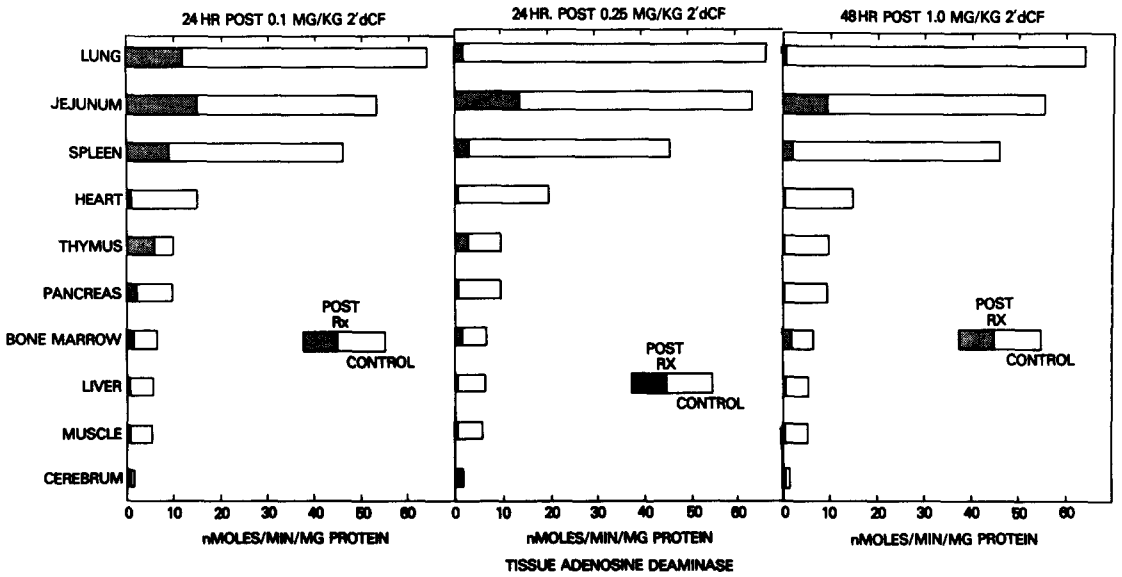


Fig. 8. Residual tissue adenosine deaminase activity after 2′-dCF. Values represent the mean of adenosine deaminase in two dogs given each dose of 2′-dCF compared to activity in two untreated dogs. Animals given 0.1 mg/kg and 0.25 mg/kg were killed at 24 hr; those given 1.0 mg/kg were killed at 48 hr.

Table 1. Residual adenosine deaminase activity in selected dog tissues 24 hr after i.v. administration of 2′-dCF*

Tissue	ADA (as per cent of control activity) Post 0.1 mg/kg 2′-dCF	Post 0.25 mg/kg 2′-dCF
Thymus	59	26
Jejunum	23	22
Pancreas	23	1
Spleen	20	6
Lung	18	3
Liver	6	6
Heart	4	0.5
Kidney	4	2
Muscle	<2	2

* Values represent the mean per cent of adenosine deaminase activity in tissues of treated dogs (two at each dose level) compared with that in tissues of two control dogs.

dose of 2′-dCF produced the greatest inhibition of enzyme activity (Table 1). However, the rapidly proliferating tissues, jejunum and bone marrow (not shown), were equally inhibited at 24 hr by 0.1 mg/kg of 2′-dCF and 0.25 mg/kg of 2′-dCF. To investigate whether this was related to initially greater enzyme inhibition with recovery over time, we studied the recovery of enzyme activity in four tissues of rats treated with 2′-dCF intraperitoneally. As seen in Fig. 9, there is an initial rapid inhibition of activity evident at 2 hr, which is maximal by 6 hr in red cells, jejunum, spleen and thymus. Activity in jejunum recovers most rapidly and completely. Activity in red cells, which do not have the capacity for new enzyme synthesis and have a long cell turnover time, recovers most slowly. It appears, therefore, that different tissues vary greatly in their rates of enzyme recovery and that the enzyme activities noted at the time of death in the dog tissues are the net result of the extent of initial inhibition and of the degree of enzyme recovery by the time of death.

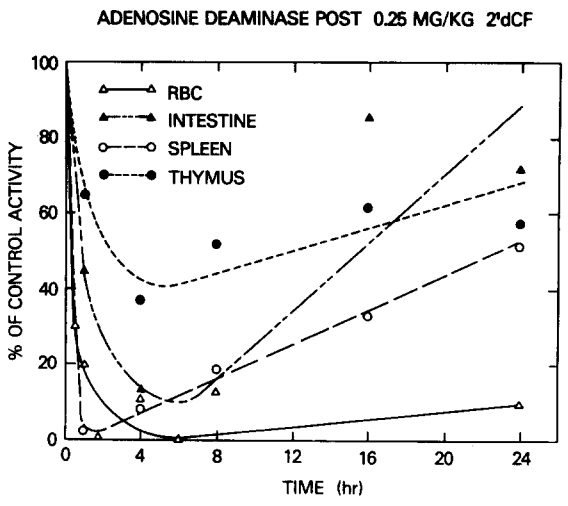


Fig. 9. Rat tissue adenosine deaminase activity after 0.25 mg/kg of 2′-dCF administered intraperitoneally. Values represent mean adenosine deaminase activity from tissues of two treated animals as a percentage of the activity in the tissues of two control animals.

DISCUSSION

This enzyme inhibition titration assay developed for the determination of 2′-dCF is modeled on similar assays for methotrexate and aminopterin [12, 13]. Cha [14, 15] and Agarwal *et al.* [16] have reviewed the problems involved in understanding the kinetics of such "tight binding" inhibitors with enzymes in general and the interaction of coformycin and 2′-dCF with adenosine deaminase in particular. Agarwal *et al.* [16] have determined the K_i for the reaction of 2′-dCF with human erythrocytic adenosine deaminase to be 2.5×10^{-12} M. The basis for this tight binding inhibition is presumed to be the resemblance of the tetrahedral C-8 carbon of 2′-dCF to the tetrahedral C-7 carbon of the transition state compound formed during the deamination of adenosine [17]. Inhibitors possessing this degree of binding have slow "on" and "off" times for the reaction with the enzyme, and equilibrium is not reached for hours. It is still possible, however, to determine 50% inhibitory concentrations of 2′-dCF of about 10^{-10} M with relatively short incubation times of 10–15 min. This allows the design of an assay that combines nanomolar sensitivity with the practical convenience of short incubation times. Thus, in our standard curve we have determined the relationship between drug concentration and degree of inhibition of enzyme activity allowing a constant period for drug–enzyme interaction, though that period is well short of the time required for equilibrium to be reached. With longer incubation times the sensitivity of the assay increases at the expense of inconvenience in time.

It should be noted that this assay depends on inhibition of enzymic activity and, therefore, will not detect drug metabolites which have lost this inhibitory property, nor will it distinguish parent compounds from metabolites which retain this property. However, no metabolites of 2′-dCF have been detected by Barondy *et al.* [9] using labeled 2′-dCF in rats. A major advantage of this assay is that it does not require radioactivity and, therefore, can be used for studies in man with unlabeled drug.

The curves for plasma concentration of 2′-dCF at times up to 6 hr exhibit bi-exponential decay. After the distribution of drug into tissues, it binds tightly to adenosine deaminase. Agarwal *et al.* [16] have demonstrated that the $T_{\frac{1}{2}}$ for the dissociation of 2′-dCF from enzyme is slow (about 29 hr). It would be expected, therefore, that the plasma curves would exhibit a γ-$T_{\frac{1}{2}}$ as well, but we have not measured the plasma drug concentration at enough late time points to estimate this value accurately.

Clearance of drug into the urine occurs at rates equal to the inulin clearance for dogs of this size. The simplest explanation is that this represents glomerular filtration solely, though a combination of tubular resorption and secretion cannot be excluded. Seventy-two to 96 per cent of administered drug could be accounted for at the time of death in either urine or tissues and this observation suggests that the role, if any, of biliary excretion is minimal, though we have not been able to measure the drug in bile because of the nature of the assay.

The tissue distribution of 2′-dCF highlights the importance of specific binding of the drug to enzyme, especially at the lower dose where tissue drug levels were proportional to tissue adenosine deaminase levels.

In man, one would expect different tissue distribution of 2′-dCF, since the human lung is not relatively rich in adenosine deaminase but human lymphoid organs and the G.I. tract are. The route of administration, i.v. or i.p. in this study, does not affect the tissue distribution of 2′-dCF. We did not study oral administration because the drug is acid-labile [18].

Those tissue effects of 2′-deoxycoformycin which are related to its inhibition of adenosine deaminase will likely be dependent on two factors, only the first of which is the level of drug attained in the tissue. The other factor is the rate at which the tissue is able to regenerate new enzyme activity, either by new cell production, new enzyme synthesis or conversion of adenosine deaminase from an uninhibited storage form of the enzyme. It follows that those tissues which rapidly replenish adenosine deaminase, by whatever means, may be less susceptible to the effects of its inhibition. However, it seems unwise to predict relative tissue toxicity solely on the basis of the extent of net enzyme inhibition, since tissues may also vary with respect to their susceptibility to the toxic effects of adenosine, deoxyadenosine and other metabolites which may accumulate as a consequence of adenosine deaminase inhibition.

The data presented here can be used to suggest some schedules for clinical trial. For use alone as an immunosuppressive agent the objective would be prolonged inhibition of lymphoid adenosine deaminase activity. Of the lymphoid tissues we examined, the thymus was relatively resistant to complete inhibition and daily doses of greater than 0.25 mg/kg or doses of 1 mg/kg every other day might be necessary. For use in combination with adenosine analogs as antitumor therapy, the relative rates of recovery of tumor and normal tissues are critical and this relationship has not been addressed in this study. However, daily doses of 0.1 to 0.25 mg/kg would allow partial recovery of activity of normal tissue between doses.

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