Biochemical Pharmacology, Vol. 16, pp. 503–507. Pergamon Press Ltd. 1967. Printed in Great Britain

EFFECTS OF 5-TRIFLUOROMETHYLDEOXYURIDINE UPON DEOXYTHYMIDINE KINASE*

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(*Received* 2 *September* 1966; *accepted* 10 *October* 1966)

Abstract—5-Trifluoromethyl-2′-deoxyuridine (F_3TdR) can be phosphorylated by deoxythymidine (TdR) kinase as easily as can the normal substrate, TdR, or the pyrimidine analog, 5-bromodeoxyuridine. F_3TdR inhibited the formation of thymidine monophosphate (d-TMP) from TdR. With 5·0 mμmoles TdR in the incubation system, the 50 per cent inhibitory concentration of F_3TdR was 10 mμmoles. The Michaelis constant, K_m, and the inhibition constant, K_i, for TdR and for F_3TdR were calculated to be $3{\cdot}3 \times 10^{-6}$ and $3{\cdot}7 \times 10^{-6}$ M. The inhibition of d-TMP formation by F_3TdR was also a complex function of the adenosine triphosphate concentration.

THE EFFICACY of the fluorinated pyrimidines, 5-fluorouracil and 5-fluoro-2′-deoxyuridine, as antitumor agents in mice[1] and in humans[2] prompted the syntheses of 5-trifluoromethyluracil (F_3T) and 5-trifluoromethyl-2′-deoxyuridine (F_3TdR).[3] The latter pyrimidine analog inhibited the growth of several mouse transplantable tumors,[4] was incorporated into the DNA of bacteriophage T4B,[5] and was mutagenic for this organism. F_3TdR was also incorporated into the DNA of cultured human cells, rendering the nucleic acid more sensitive to ultraviolet and X-irradiation.[6] The latter studies implied the prior formation of the nucleotide of the pyrimidine analog.

At the biochemical level, F_3TdR inhibited the phosphorolytic cleavage of fluorodeoxyuridine by nucleoside phosphorylase.[7] The phosphorylated derivative, F_3d-TMP, inhibited thymidylate synthetase.[8, 9]

Recently, the enzyme deoxythymidine kinase has been obtained in a partially purified form, and some of its properties have been determined.[10] The enzyme has a fairly broad substrate specificity. The enzyme will catalyze the phosphorylation of deoxythymidine, 5-bromodeoxyuridine, 5-iododeoxyuridine, and 5-chlorodeoxyuridine to the same extent; deoxyuridine and 5-fluorodeoxyuridine are phosphorylated to a lesser degree. The present study was devised to ascertain (1) whether F_3TdR could be phosphorylated with the aid of deoxythymidine kinase, and (2) whether the pyrimidine analog would inhibit the formation of deoxythymidine monophosphate (d-TMP) from deoxythymidine (TdR). The results are reported in this manuscript.

MATERIALS AND METHODS

Chemicals. ATP-8-^{14}C (30 μc/μmole) and TdR-2-^{14}C (30 μc/μmole) were obtained

* Supported by Research Grant CA06571 from the United States Public Health Service.
† Recipient of a Lederle Medical Faculty Award, 1966–68.

from Schwartz BioResearch, Inc., and New England Nuclear Corp. respectively. F_3TdR was a product of the Mann Research Laboratories.

Enzyme. Male Cheek–Jones rats (150–170 g) were partially hepatectomized according to the procedure of Higgins and Anderson[11] and sacrificed 24 hr later. The regenerating liver was removed, washed in 0·9% saline, and homogenized in 0·25 M sucrose (1:4·5, w/v). The homogenate was centrifuged at 100,000 *g* for 60 min, and deoxythymidine kinase activity was concentrated from the soluble preparation as described in previous publications.[10, 12] The final enzyme preparation had a specific activity of approximately 50 mμmoles d-TMP formed per 15 min/mg protein.

Enzyme assay. TdR kinase was assayed in the following system: enzyme, approximately 7 μg protein; ATP, 5 mM; $MgCl_2$, 2·5 mM; TdR-2-^{14}C, 0–0·2 μc; and 0·05 M Tris buffer, pH 8·0, in a total volume of 0·25 ml. The incubation was conducted in test tubes at 37° for 15 min. Aliquots of 25 μl were removed, placed on disks of DEAE-cellulose, and the amount of d-TMP was determined as described previously.[10, 13, 14]

The concentration of protein in the enzyme extracts was estimated by the method of Lowry *et al.*[15]

The substrate specificity was determined by employing ATP-8-^{14}C, 0·1 μc, as the radioactive precursor. The pyrimidine deoxyribonucleosides were present at $4·0 \times 10^{-5}$ M. The ADP-8-^{14}C was determined after an electrophoretic separation in 0·1 M citrate buffer, pH 5·2, at 900 V for 2 hr.

RESULTS

Phosphorylation of F_3TdR

The pyrimidine analog, F_3TdR, was phosphorylated in the presence of TdR kinase and ATP (Table 1). The amount of ADP formed was comparable when deoxythymidine, 5-bromodeoxyuridine, or F_3TdR was employed as acceptor. Deoxyuridine, as in previous studies,[10] was an inferior acceptor.

TABLE 1. SUBSTRATE SPECIFICITY OF TdR KINASE

Acceptor	ADP Formed (mμmoles)
Deoxythymidine	0·40
5-Bromodeoxyuridine	0·39
Deoxyuridine	0·26
F_3TdR	0·40

TdR kinase (regenerating liver), 5 μg protein, was incubated with: $MgCl_2$, 2·5 mM; ATP-8-^{14}C, 0·4 mM (0·1 μc); pyrimidine deoxyribonucleosides, 0·4 mM; 0·05 M Tris buffer, pH 8·0, to make a total volume of 0·25 ml. The incubation was conducted at 37° for 15 min. The ADP-^{14}C was separated by electrophoresis in 0·1 M citrate buffer, pH 5·2, at 900 V for 2 hr.

Inhibition of deoxythymidine kinase by F_3TdR

The inhibition of TdR kinase by F_3TdR is indicated in Fig. 1. A progressively increasing inhibition was apparent with increasing concentration of F_3TdR; the

concentration of inhibitor yielding 50 per cent of the control velocity was 10 mμmoles/0·25 ml reaction system.

Double-reciprocal plots for TdR kinase and its inhibition by F_3TdR

The inhibition of TdR kinase by F_3TdR was analyzed according to the method of Lineweaver and Burk.[16] The K_m for TdR was $3{\cdot}3 \times 10^{-6}$ M and the V_m was 1·2 moles/hr/g protein. These values are in good agreement with those published previously.[10] The inhibition of TdR kinase by F_3TdR was competitive (Fig. 2), and the

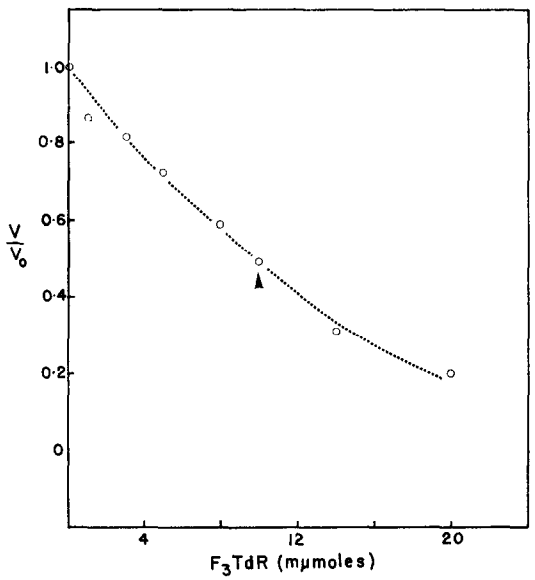

FIG. 1. Inhibition of TdR kinase by F_3TdR. The reaction mixture for the assay of TdR kinase included: enzyme, 15·0 μg; TdR-2-^{14}C, 5·0 mμmoles (0·1 μc); ATP, 5 mM; $MgCl_2$, 2·5 mM; F_3TdR; 0·05 M Tris buffer, pH 8·0, to 0·25 ml. V_0 = mμmoles d-TMP produced in the control system in 15 min. V = mμmoles d-TMP/15 min in inhibited system. V_0 = 2·40 mμmoles/15 min.

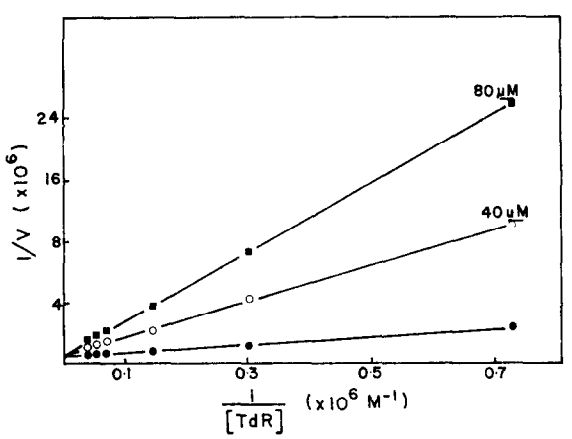

FIG. 2. Double-reciprocal plots for TdR kinase and its inhibition by F_3TdR. The incubation system is given in the legend to Fig. 1 with the following exceptions; the concentration of TdR was varied; F_3TdR concentration is presented in the figure; the incubation was shortened to 10 min; enzyme, 7·9 μg. V = moles d-TMP/l./15 min.

inhibition constant, K_i, was calculated as $3{\cdot}7 \times 10^{-6}$ M, a value approximating the Michaelis constant for TdR.

The inhibition of TdR kinase by F_3TdR was analyzed as a function of the ATP concentration. It had previously been shown[10, 12] that the inhibition of TdR kinase by the end-product inhibitor, deoxythymidine triphosphate, was also related to the concentration of ATP. The inhibition of TdR kinase by F_3TdR proved to be a complex function of the ATP concentration (Fig. 3). The shape of the curves obtained from the

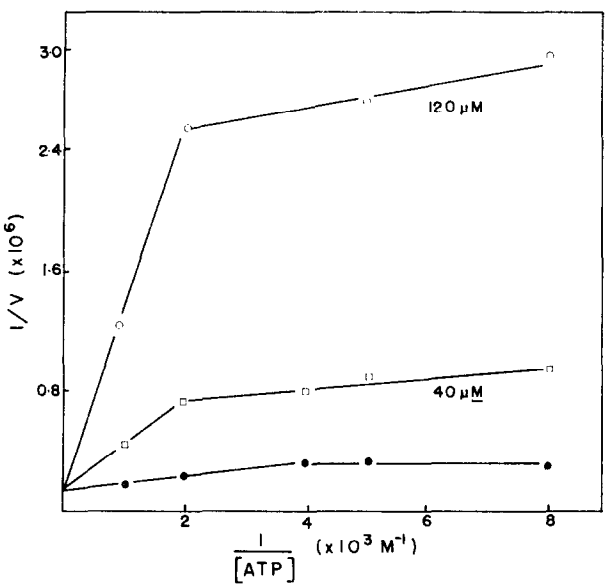

FIG. 3. Inhibition of TdR kinase by F_3TdR as a function of [ATP]. See the legend to Fig. 1. The [TdR] was $1{\cdot}3 \times 10^{-5}$ M; the protein content of the enzyme was 7·9 μg; the [F_3TdR] is indicated on the figure; the incubation was for 15 min. V = moles d-TMP/l./15 min.

data with the inhibited systems were quite similar to that obtained from the control system. The inhibition by F_3TdR was more pronounced at the higher concentrations of ATP.

DISCUSSION

The results of this report clearly indicate the phosphorylation of F_3TdR by purified TdR kinase of regenerating rat liver. Presumably the monophosphorylated derivative can be readily converted to the triphosphate via thymidylate and thymidine diphosphate kinases. Since F_3TdR can eventually find its way into the DNA of cultured human cells and of bacteriophage T4B, presumably trifluorodeoxythymidine triphosphate can serve as substrate for the enzyme DNA polymerase. In these respects, F_3TdR resembles the other thymidine analogs, 5-bromodeoxyuridine and 5-iododeoxyuridine.[17, 18] Unlike 5-bromodeoxyuridine, however, F_3TdR after conversion to F_3d-TMP can inhibit mammalian thymidylate synthetase, and in this respect closely resembles the 5-fluoro component.[9]

Not only can F_3TdR serve as substrate for the enzyme TdR kinase, the pyrimidine analog can also inhibit the conversion of deoxythymidine to deoxythymidine monophosphate by this enzyme. The inhibition constant is of the same order of magnitude as the Michaelis constant for the true substrate, deoxythymidine. The reported Van der Waals radius for the trifluoromethyl group at the 5-position of the pyrimidine, 2·44 Å,[5] is close to the Van der Waals radius for the methyl group, i.e. 2·0 Å. The size of the chloro, bromo, and iodo atoms, with radii of 1·80, 1·95 and 2·15 Å, respectively, is also similar to that of the methyl group. These pyrimidine deoxyribonucleosides can all serve with equal facility as substrates for TdR kinase.[10] On the other hand, the smaller atoms, hydrogen and fluorine, in the 5-position of the pyrimidine, with Van der Waals radii of 1·2 and 1·35 Å, respectively, are not very good substrates for the enzyme nor do they inhibit the formation of d-TMP from TdR to any great extent. It is interesting that the increased acidity of F_3TdR, i.e. $pK_a = 7{\cdot}35$ while the pK_a for thymine $= 9{\cdot}82$,[5] does not influence the extent of the reaction of F_3TdR with the enzyme. From the data of this paper one could expect that F_3TdR would be an inhibitor of systems that possess enhanced levels of TdR kinase, e.g. rapidly proliferating tumors,[10, 13, 19] and indeed this is the case.[4]

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