

The Antitumor Mechanism of 1-(2-Deoxy-2-fluoro-4-thio- β -D-arabinofuranosyl)-cytosine: Effects of Its Triphosphate on Mammalian DNA Polymerases

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The mechanism of action of the antitumor nucleoside analog 1-(2-deoxy-2-fluoro-4-thio- β -D-arabinofuranosyl)cytosine (4'-thio-FAC) was investigated. 4'-Thio-FAC inhibited cellular DNA synthesis, but not RNA and protein syntheses. We observed potent inhibitory action of the triphosphate of 4'-thio-FAC (4'-thio-FACTP) against DNA polymerase α , whereas it showed moderate inhibition of DNA polymerase β and little inhibition of DNA polymerase γ . The kinetic analysis showed that the inhibition mode of 4'-thio-FACTP towards DNA polymerase α was mixed type, implying a chain-terminating effect of 4'-thio-FACTP. The triphosphate of 2'-deoxy-2',2'-difluorocytidine (gemcitabine), a known antitumor nucleoside, did not show potent inhibition of these three DNA polymerases. Thus, the effect of the diphosphate of gemcitabine on ribonucleotide reductase was suggested to be more important for the antitumor action of gemcitabine. From these findings, the main target enzymes of 4'-thio-FAC and gemcitabine appear to be different. We found a synergistic effect of the two drugs in an *in vitro* model, which supports the above idea.

Key words: 4'-Thio-FAC — DNA polymerase — Gemcitabine — Mitochondria toxicity — Antitumor mechanism

1-(2-Deoxy-2-fluoro-4-thio- β -D-arabinofuranosyl)cytosine (4'-thio-FAC) is a very promising agent because of its potent antitumor activity upon oral administration.¹⁾ It is a deoxycytidine analog similar to 1- β -D-arabinofuranosylcytosine (araC) or 2'-deoxy-2',2'-difluorocytidine (gemcitabine), which is used against leukemia or solid cancers. The *in vitro* antitumor spectra of 4'-thio-FAC, gemcitabine and araC against a human cancer cell line panel were similar as reported previously.²⁾ Therefore, 4'-thio-FAC was expected to inhibit DNA synthesis, like araC and gemcitabine. However, the antitumor mechanism of 4'-thio-FAC has not yet been clarified in detail.

DNA polymerase, which plays a major role in DNA replication and repair, is one of the most important target molecules of antitumor nucleosides. There are several subtypes of eukaryotic DNA polymerase and their localization and function have been studied.³⁾ In DNA replication, DNA polymerases α and δ have been implicated in the synthesis of lagging and leading strands, respectively, whereas DNA polymerases β and ϵ have been suggested to work in DNA repair. DNA polymerase γ is encoded in the nucleus but is localized in the mitochondria, and is responsible for the mitochondrial DNA replication. Recently, several antiviral nucleoside analogs, including 3'-azido-3'-deoxythymidine (zidovudine, AZT), 2',3'-di-

deoxycytidine (zalcitabine, ddC) and 1-(2-deoxy-2-fluoro-1- β -D-arabinofuranosyl)-5-iodouracil (fialuridine, FIAU), were reported to inhibit DNA polymerase γ after intracellular phosphorylation, causing mitochondria dysfunction and severe chronic toxicity in particular organs.⁴⁾

Thus, we tested the inhibitory activity of the triphosphate of 4'-thio-FAC on DNA polymerases α , β and γ not only to clarify the antitumor mechanism, but also to assess its potential for mitochondrial toxicity. The differences in the mechanisms of action between 4'-thio-FAC and gemcitabine are discussed.

MATERIALS AND METHODS

Drugs and reagents 4'-Thio-FAC and gemcitabine were synthesized by us as described previously.^{5,6)} The 5'-triphosphate of 4'-thio-FAC (4'-thio-FACTP) and gemcitabine (dFdCTP) were also synthesized by us. The 5'-triphosphate of ddC (ddCTP) and 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). AraC was from Yamasa Corp. (Choshi). [Methyl-³H]thymidine (83.2 Ci/mmol), [5-³H]uridine (23.1 Ci/mmol) and [4,5-³H]L-leucine (47.5 Ci/mmol) were obtained from DuPont NEN (Boston, MA).

Cell culture Oral epidermoid carcinoma KB was derived from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Moregate Laborato-

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ries, Melbourne, Australia), 100 units/ml of penicillin, and 0.1 $\mu\text{g}/\text{ml}$ of streptomycin.

Inhibitory effects of nucleosides on thymidine, uridine and leucine incorporation into macromolecules KB cells were seeded at a density of 2.5×10^4 cells/well in a 96-well microplate. After incubation at 37°C in 5% CO_2 incubator for 24 h, the drug solution was added to each well. After further incubation for 3 h, the medium was removed and the cell monolayer was washed once with drug-free medium. Then, a medium containing [^3H]thymidine (0.2 $\mu\text{Ci}/\text{well}$), [^3H]uridine (0.2 $\mu\text{Ci}/\text{well}$), or [^3H]leucine (0.5 $\mu\text{Ci}/\text{well}$) was added to each well. After incubation at 37°C for 2 h, the medium was removed and the cells were trypsinized and harvested onto glass filter paper using a "Titertek" cell harvester 550 (Flow Laboratories, Irvine, UK). Filters were washed with water five times, then dried, and their radioactivity was counted in a scintillation liquid (ACS II, Amersham, Buckinghamshire, UK). The inhibitory effects of the drugs are shown as percent of incorporation inhibited compared with the control values.

Preparation and enzyme assay of DNA polymerases α , β and γ DNA polymerases α and β were purified from calf thymus,⁷⁾ and DNA polymerase γ was purified from bovine liver according to the method described previously.⁸⁾

DNA polymerase α activity was measured in a mixture (25 μl) containing 40 mM potassium phosphate (pH 7.4), 100 $\mu\text{g}/\text{ml}$ of activated calf thymus DNA, 40 μM each of dCTP, dATP and dGTP, 5 μM of [methyl- ^3H]TTP (4 Ci/mmol), 1 mM dithiothreitol, 8 mM MgCl_2 and an aliquot of the enzyme solution. In the case of DNA polymerase β , potassium phosphate was replaced by 40 mM Tris-HCl

(pH 8.5). In the case of DNA polymerase γ , 200 mM KCl was added to the mixture for the assay of DNA polymerase α . Incubation was carried out at 37°C for 10 min. For kinetic analysis, the concentration of the inhibitor and dCTP were varied.

After incubation, the reaction mixture was chilled in an ice bath and 20 μl was transferred to DEAE-cellulose paper discs (DE81, Whatman, Kent, UK). The discs were washed with 5% Na_2HPO_4 (six times), water (once), ethanol (twice) and ether, and dried. The remaining radioactivity was measured in a scintillation liquid (ACS II).

Inhibitory effects of the drugs on cancer cell growth The MTT assay⁹⁾ with some modifications¹⁾ was used to determine the inhibitory effects of drugs on cell growth after 72 h continuous exposure. The combination effects of 4'-thio-FAC and gemcitabine were analyzed using the isobologram method of Steel and Peckham.¹⁰⁾

RESULTS

Inhibitory effects of 4'-thio-FAC on thymidine, uridine and leucine incorporation into macromolecules in KB cells

We first determined the effects of 4'-thio-FAC on cellular DNA, RNA, and protein syntheses by measuring the incorporation of labeled thymidine, uridine and leucine into the macromolecular fraction of KB cells. 4'-Thio-FAC potentially inhibited cellular DNA synthesis in a dose-dependent manner (Fig. 1). At concentrations of 0.2 and 2 μM , it caused above 90% inhibition of DNA synthesis. However, RNA and protein syntheses were not inhibited greatly by 4'-thio-FAC even at a concentration of 2 μM . The representative antitumor nucleosides gemcitabine and araC also inhibited DNA synthesis in a dose-dependent manner, like

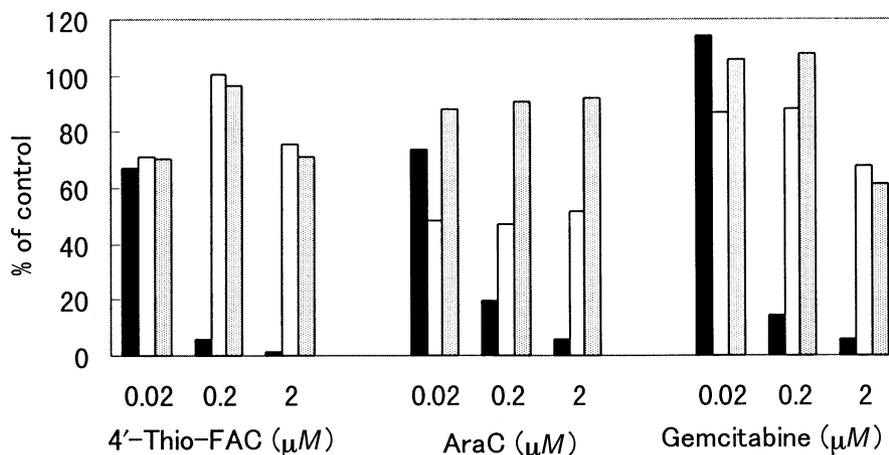


Fig. 1. The inhibitory effects of 4'-thio-FAC, araC and gemcitabine on the incorporation of [^3H]thymidine (closed bar), [^3H]uridine (open bar) and [^3H]leucine (dotted bar) into the macromolecular fraction of KB cells. Each value represents the average of triplicate determinations.

4'-thio-FAC (Fig. 1). AraC inhibited even RNA synthesis at concentrations of 0.02, 0.2 and 2 μM , though the inhibition was not dose-dependent.

Inhibitory effects of 4'-thio-FACTP, dFdCTP and ddCTP on mammalian DNA polymerases α , β and γ

Since DNA polymerases are essential proteins for DNA synthesis and many triphosphates of antimetabolite nucleosides are known to inhibit DNA polymerase, we examined the inhibitory effects of 4'-thio-FACTP on mammalian DNA polymerases α and β purified from calf thymus and γ from bovine liver. As shown in Fig. 2, 4'-thio-

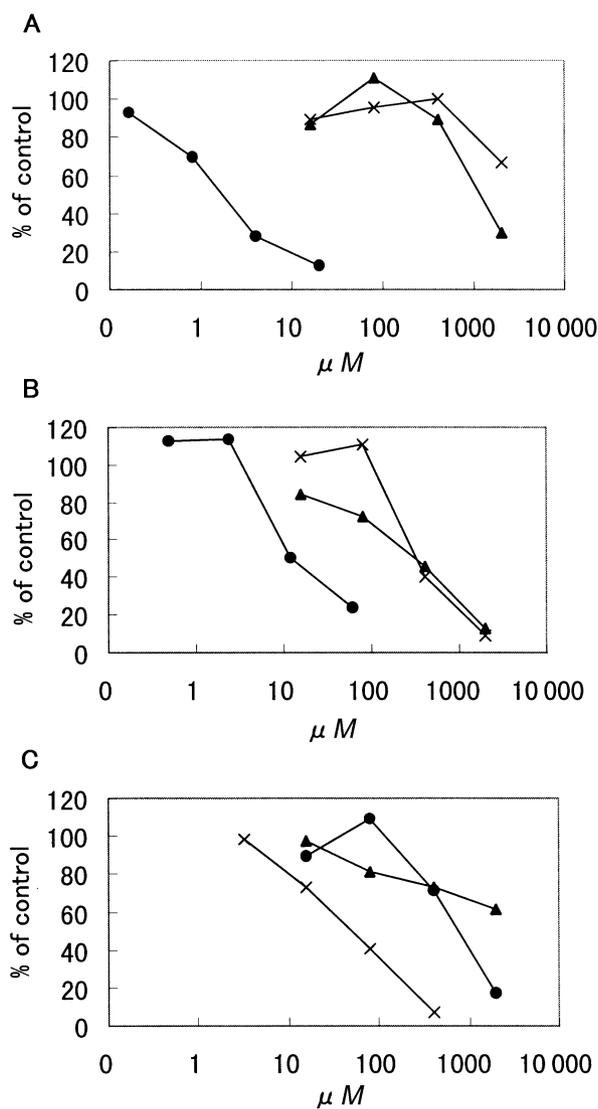


Fig. 2. The inhibitory effects of 4'-thio-FACTP, dFdCTP and ddCTP on mammalian DNA polymerases α (A), β (B) and γ (C). Each value represents the average of duplicate determinations. ● 4'-thio-FACTP, ▲ dFdCTP, × ddCTP.

FACTP showed a potent inhibition of DNA polymerase α ; the IC_{50} value was 1.9 μM . It also inhibited DNA polymerase β , showing a ten-fold larger IC_{50} value (19 μM) than that against DNA polymerase α . It was noteworthy that the inhibitory effect of 4'-thio-FACTP on DNA polymerase γ was very weak; the IC_{50} value was 690 μM . To compare the effects of 4'-thio-FACTP with that of the triphosphate of a known antitumor nucleoside, we also examined the inhibitory effect of dFdCTP. Surprisingly, its effects against DNA polymerases α , β and γ were very weak (IC_{50} values were >2000, 450 and >2000 μM , respectively). The inhibitory effects of ddCTP, which was reported to inhibit DNA polymerase γ ,¹¹⁾ on these polymerases were also examined. It inhibited DNA polymerase γ with an IC_{50} value of 46 μM , whereas it did not show potent inhibition of DNA polymerase α or β (IC_{50} values were 1200 and 240 μM , respectively).

Kinetic analysis of the inhibition of DNA polymerase α by 4'-thio-FACTP

The inhibition mode of DNA polymerase α by 4'-thio-FACTP was determined using a

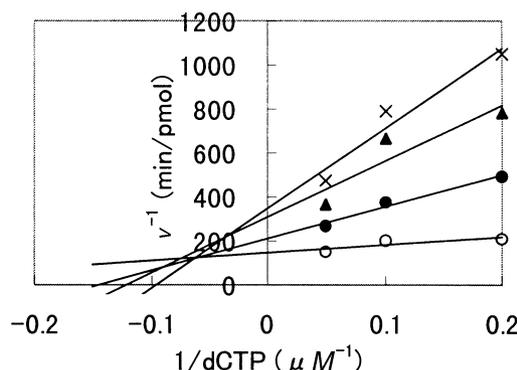


Fig. 3. Kinetic analysis of the inhibition of DNA polymerase α by 4'-thio-FACTP. Calf thymus DNA polymerase α activity was assayed as described in "Materials and Methods" in the presence of 0 (○), 0.2 (●), 0.5 (▲) or 1 μM (×) 4'-thio-FACTP, and a Lineweaver-Burk plot of the data was prepared.

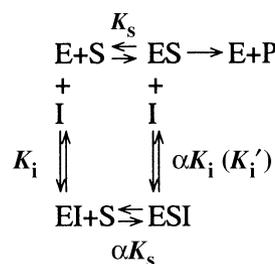


Fig. 4. Proposed scheme for mixed inhibition of the enzyme. Parameters are explained in the text.

kinetic assay. The Lineweaver-Burk plot showed mixed inhibition kinetics¹²⁾ with dCTP (Fig. 3). This is consistent with the scheme in Fig. 4 where E, S, P, I, K_s and K_i (K_i') refer to the enzyme, substrate, product, inhibitor, binding constant of the substrate to the enzyme and dissociation constant for the EI (ESI), respectively. The equation that can be derived from the scheme in Fig. 4 is:

$$v = \frac{V_{\max}[S]}{K_m(1+[I]/K_i)+[S](1+[I]/K_i')}$$

where v , $[S]$, $[I]$, V_{\max} and K_m are the initial reaction velocity, substrate concentration, inhibitor concentration, maximum velocity and Michaelis constant, respectively. The K_i and K_i' , determined from replots of the reciprocal of the vertical axis intercept against the inhibitor concentration and replots of the reciprocal of the slope against the inhibitor concentration, were 0.10 and 0.44 μM , respectively.

Analysis of the combination effects of 4'-thio-FAC and gemcitabine on cancer cell growth From the findings mentioned above, the target enzyme of 4'-thio-FAC was considered to be DNA polymerase α , whereas that of gemcitabine was not. Thus, we tested the synergistic effect of 4'-thio-FAC and gemcitabine. The cytotoxicity of both drugs against KB cells was determined alone or in combination, and their synergism was analyzed using the isobologram method.¹⁰⁾ The plotted IC_{50} values were in the area to the left of the envelope of additivity on the isobologram, confirming synergistic cytotoxic effects of the two drugs in combination (Fig. 5).

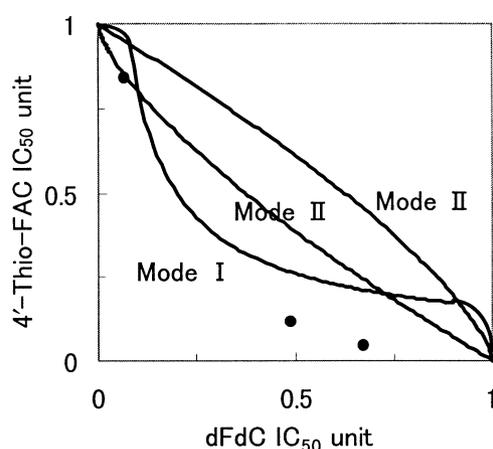


Fig. 5. Isobologram analysis of the effects of combination of 4'-thio-FAC and gemcitabine on KB cell growth. On the basis of the concentration-effect curve findings of each drug, the isoeffect curves (mode I and mode II) were drawn and an "envelope of additivity" was obtained as described previously.¹⁰⁾ The experimental findings of the concentrations of each drug which gives 50% inhibition in combination with the other were plotted as the IC_{50} unit, the relative value to the IC_{50} of each drug alone.

DISCUSSION

The antitumor mechanism of 4'-thio-FAC was investigated and compared with those of other antitumor nucleosides. It was shown that 4'-thio-FAC inhibited DNA synthesis in a dose-dependent manner (Fig. 1). Gemcitabine and araC also inhibited DNA synthesis. AraC showed about 50% inhibition of RNA synthesis at concentrations of 0.02, 0.2 and 2 μM , though the inhibition was not dose-dependent. Though it was not clear why araC inhibited RNA synthesis at low concentration, the inhibition of RNA synthesis at high concentration was thought to be a secondary effect of DNA inhibition. Thus, these three nucleosides inhibit cellular DNA synthesis.

Many antitumor agents cause side-effects, including myelosuppression and intestinal dysfunction. Such side-effects are relatively early-appearing and often limit the doses of the agents. In addition, the attention must also be given to late-appearing side-effects because the term of therapy tends to be prolonged in adjuvant chemotherapy after surgery. Since 4'-thio-FAC shows a good chemotherapeutic effect upon oral treatment, it is expected to be used for adjuvant chemotherapy. Its toxicity in long-term treatment must be low for this use. FIAU, an antiviral agent which has a fluoridated carbohydrate moiety, like 4'-thio-FAC and gemcitabine, has been reported to cause fatal liver toxicity in long-term therapy.¹³⁾ This toxicity was suggested to be related to mitochondrial dysfunction via FIAU inhibition of DNA polymerase γ after intracellular phosphorylation.¹⁴⁾ Therefore, we tested 4'-thio-FAC for its inhibitory effect on the three DNA polymerases and found that it showed potent inhibition of DNA polymerase α , whereas it showed moderate inhibition of DNA polymerase β and little inhibition of DNA polymerase γ ; the ratio of the IC_{50} value against DNA polymerase γ to that against DNA polymerase α was >300 (Fig. 2). 4'-Thio-FAC inhibited DNA synthesis in KB cells, but not RNA and protein syntheses (Fig. 1). Therefore, 4'-thio-FAC must be phosphorylated at the 5'-hydroxyl moiety and converted to 4'-thio-FACTP, which potently inhibits DNA polymerase α , resulting in inhibition of cellular DNA synthesis and tumor growth arrest. If the dose of 4'-thio-FAC was maintained at an adequate level for inhibiting DNA polymerase α in tumor tissue, DNA polymerase γ in normal tissue would not be inhibited because of the difference between the IC_{50} values for DNA polymerases α and γ . Thus, the incidence of the mitochondrial toxicity due to 4'-thio-FAC treatment would be low. An anti-human immunodeficiency virus agent, ddC, was reported to reduce mitochondrial DNA content and induce peripheral neuropathy.^{15, 16)} In the present study, ddCTP inhibited calf liver DNA polymerase γ , although it showed little inhibition of DNA polymerases α and β (Fig. 2). It has been suggested that ddC suppresses human immunodeficiency

virus (HIV) replication by inhibiting reverse transcriptase activity.¹⁷⁾ Possibly, ddCTP might inhibit not only HIV reverse transcriptase, but also DNA polymerase γ in neural tissue of the patients. On the other hand, the effects of dFdCTP on DNA polymerases α , β and γ were weak (Fig. 2). A previous study revealed weak competitive inhibition of dFdCTP on DNA polymerases α and ϵ .¹⁸⁾ In the present and several other studies,^{19–21)} the 5'-diphosphate of gemcitabine (dFdCDP), which had been shown to inhibit ribonucleotide reductase, was also suggested to be responsible for the antitumor effect of gemcitabine. Thus, 4'-thio-FAC may strongly inhibit DNA polymerase α after conversion to its triphosphate, whereas metabolites of gemcitabine may have several sites of action. AraC's action on DNA replication is reported to be mediated through DNA polymerase α .²²⁾ Thus, it is suggested that the antitumor action of 4'-thio-FAC and araC is essentially the same. However, 4'-thio-FAC exhibits potent inhibition against solid tumors and its toxicity is low *in vivo*,^{1,2)} whereas araC is ineffective against solid tumors. They are thought to differ in absorption, distribution, metabolism and excretion characteristics in the animal body. 4'-Thio-FAC is more resistant to cytidine deaminase than araC.¹⁾ Further studies are needed to explain the difference between the effects of two drugs.

To clarify the mechanism of 4'-thio-FACTP inhibition of DNA polymerase α , we analyzed the kinetics of the inhibition. Lineweaver-Burk plot analysis indicated that the inhibition mode was mixed type (Fig. 3). Therefore, it was suggested that 4'-thio-FACTP inhibited DNA poly-

merase α in two ways: (a) it retarded the DNA chain elongation by competing with dCTP at the nucleotide binding site of DNA polymerase α , and (b) it was incorporated into DNA and terminated chain elongation, and the resulting short-length DNA chains inhibited DNA polymerase α non-competitively with dCTP. Additional experiments to identify the short DNA products will be required to confirm this hypothesis.

Although both 4'-thio-FAC and gemcitabine inhibited DNA synthesis (Fig. 1), the possibility remains that the main target enzymes of their active metabolites are different. Therefore, the combination of 4'-thio-FAC and gemcitabine would be expected to have a synergistic chemotherapeutic effect. In an *in vitro* model using human epidermoid carcinoma KB cells, a synergistic effect was indeed observed (Fig. 5). For clinical application of this combination therapy, more information is required about the effect on other cell lines, including lung or pancreas tumor cells, because gemcitabine is administered to patients suffering from these cancers.

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