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ORIGINAL ARTICLE

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Effects of gemcitabine and araC on in vitro DNA synthesis mediated by the human breast cell DNA synthesome

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Abstract *Purpose*: Gemcitabine (dFdC) and cytarabine (araC) are both analogs of deoxycytidine. Gemcitabine is a relatively new drug that has been shown in both clinical trials and in vitro systems to have more potent antitumor activity than araC. We have previously isolated a fully functional multiprotein DNA replication complex from human cells and termed it the DNA synthesome. Using the DNA synthesome, we have successfully examined the mechanism of action of several anticancer drugs that directly affect DNA synthesis. In this study, we compared the effects of dFdC and araC on in vitro DNA synthesis mediated by the DNA synthesome with the effects of these drugs on intact MCF7 cell DNA synthesis. *Methods*: We examined the effects of dFdC and araC on intact MCF7 cell DNA synthesis and clonogenicity. We also performed in vitro SV40 replication assays mediated by the MCF7 cell-derived DNA synthesome in presence of dFdCTP and araCTP. The types of daughter molecules produced in the assay were analyzed by neutral and alkaline agarose gel electrophoresis. Finally, we examined the effects of dFdCTP and araCTP on the synthesome-associated DNA polymerase α and δ activities. *Results*: Our results showed that dFdC was more potent than araC at inhibiting intact MCF7 cell DNA synthesis and clonogenicity. [^{3}H]Thymidine incorporation was inhibited by 50% at a dFdC concentration of 10 μM, which was about tenfold lower than the concentration of araC required to inhibit intact cell DNA synthesis by the same amount. As examined by clonogenicity assay, dFdC was also significantly more cytotoxic than araC after a 24-h incubation. In vitro SV40 replication assays using the DNA synthesome derived from MCF7 cells demonstrated that the formation of full-length DNA along with replication intermediates were inhibited by dFdCTP in a concentration-dependent manner. Full-length DNA was produced in the in vitro DNA replication assay even when the dFdCTP was incubated in the assay at concentrations of up to 1 mM. We observed that in the presence of 10 μM dCTP, 3 μM dFdCTP and 60 μM araCTP were required to inhibit in vitro SV40 DNA synthesis by 50%. Although dFdCTP is more potent than araCTP at inhibiting in vitro SV40 DNA synthesis, there was no significant difference between the inhibitory effect of these two drugs on the activity of the MCF7 synthesome-associated DNA polymerases α and δ. It was found that the drug concentrations required to inhibit 50% of the synthesome-associated DNA polymerase δ activity were much higher than those required to inhibit 50% of DNA polymerase α activity for both dFdCTP and araCTP. *Conclusion*: Taken together, our results demonstrated that: (1) dFdC is a more potent inhibitor of intact cell DNA synthesis and in vitro SV40 DNA replication than araC; (2) the decrease in the synthetic activity of synthesome-mediated in vitro SV40 origin-dependent DNA synthesis by dFdCTP and araCTP correlates with the inhibition of DNA polymerase α activity; and (3) the MCF7 cell DNA synthesome can serve as a unique and relevant model to study the mechanism of action of anticancer drugs that directly affect DNA synthesis.

Key words AraC · Gemcitabine · In vitro · DNA replication · DNA synthesome

Abbreviations *ara-C* 1-β-D-arabinofuranosylcytosine · *ara-CTP* 1-β-D-arabinofuranosylcytosine triphosphate · *dFdC* 2′,2′-difluorodeoxycytidine, gemcitabine ·

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dFdCTP gemcitabine triphosphate · *DTT* dithiothreitol · *EDTA* ethylenediaminetetraacetic acid · *EGTA* ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid · *HEPES N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) · *PBS* phosphate-buffered saline · *PMSF* phenylmethyl sulfonyl fluoride · *SV40* simian virus 40 · *SDS* sodium dodecyl sulfate · *Tris* tris(hydroxymethyl) aminoethane

Introduction

Nucleoside antimetabolites comprise one of the most effective classes of drugs used in the treatment of viral diseases and specific types of malignancy. The biological activity of most nucleoside antimetabolites is due to their ability to inhibit the DNA synthetic process, which is an essential function both for cell division and proliferation. 1-β-D-Arabinofuranosylcytosine (araC) has been used effectively in the clinic to treat hematologic cancers [6]. 2′,2′-Difluorodeoxycytidine (gemcitabine, dFdC) is a novel deoxycytidine analog with structural and metabolic similarities to araC. Both of these compounds are prodrugs which are transported into the cell where they are activated following phosphorylation by deoxycytidine kinase. AraC and dFdC differ in structure from the parent nucleoside, deoxycytidine, by specific modifications to the 2′ carbon of the furanose ring. Clinical trials have shown that dFdC is effective in most solid tumors and more potent and less toxic than araC [11, 13, 14, 19, 21]. Studies of intact cells have indicated that inhibition of DNA synthesis is the predominant effect of dFdC and araC [16, 17, 26, 27, 29]. Like araC, the major targets for dFdCTP are the DNA polymerases. It has been shown that incorporation of araCTP and dFdCTP into DNA is most likely the primary mechanism by which these drugs exert their cytotoxic effects [27]. Using in vitro DNA primer extension assays employing purified DNA polymerases, dFdCTP and araCTP have shown qualitative and quantitative differences in their molecular actions on DNA synthesis [16]. Studies comparing dFdC and araC have shown that dFdC is transported more rapidly and is a better substrate for deoxycytidine kinase than araC [11]. Furthermore, dFdC inhibits ribonucleotide reductase and thus causes depletion of intracellular nucleotide pools [12]. However, araC is not known to inhibit the activity of ribonucleotide reductase [12]. Finally, dFdCTP also shows a slower elimination rate than araCTP [11]. All of these characteristics of dFdC result in the development of higher intracellular concentrations of active metabolite (i.e. dFdCTP). Although inhibition of DNA synthesis has been strongly correlated with intracellular dFdCTP concentration [12], little work has been done to directly compare the effects of dFdCTP and araCTP on reducing the level of DNA replication within the cell.

We have previously reported that a highly purified multiprotein form of DNA polymerase (the DNA synthesome) can be isolated from a variety of mammalian cell types and tissues [2, 3, 15, 18, 22, 31, 34]. We have shown that the DNA synthesome is fully competent to support origin-specific large T antigen-dependent in vitro SV40 DNA replication [2, 3, 22, 34]. Biochemical characterization of the DNA synthesome has identified several protein components of the complex that are essential for DNA replication [15, 23]. These proteins include the DNA polymerases α, δ, and ε, DNA primase, topoisomerases I and II, proliferating cell nuclear antigen, replication factor C, replication factor A, DNA helicase, and DNA ligase I [2, 24, 34]. Most importantly, in the presence of viral large T antigen and the SV40 replication origin sequence, the synthesome is fully competent to carry out all phases of the DNA replication process required to replicate an SV40 origin-containing plasmid in vitro. We have successfully examined the action of araC and camptothecin using this model system [1, 4, 10, 32, 33] and have now extended the results of these studies by exploring how the inhibitory effects of dFdC and araC compare with one another.

Our studies compared the inhibitory effects of dFdC and araC on intact human breast cancer cell DNA synthesis and in DNA synthesis mediated by our in vitro DNA replication assay system. In comparison with araC, dFdC was shown to more strongly inhibit the in vitro DNA synthetic activity of the DNA synthesome and the intact MCF7 cells DNA synthesis. We also demonstrated that the inhibition of in vitro DNA synthesis was preferentially mediated by the action of dFdCTP and araCTP on DNA polymerase α and not DNA polymerase δ.

Materials and methods

Materials

AraC and araCTP were purchased from Sigma Chemical Co. (St. Louis, Mo.). dFdC and dFdCTP were kindly supplied by Eli Lilly & Co. (Indianapolis, Ind.). AraC and dFdC were dissolved in water. AraCTP and dFdCTP were dissolved in 10 m*M* HEPES, pH 7.5. The stock solutions were aliquoted and stored at −80 °C. [α-^{32}P]dGTP (3000 Ci/mmol, 10 mCi/ml), [methyl-^{3}H]thymidine (90 Ci/mmol; 2.5 mCi/ml) and [^{3}H]dTTP (72.6 Ci/mmol) were obtained from Dupont New England Nuclear (Boston, Mass.).

Cell culture

Suspension cultures of MCF7 cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of calf and fetal bovine serum. Exponentially growing cells were harvested and washed three times with phosphate-buffered saline (PBS). The cells were then pelleted by low-speed centrifugation. The cell pellets were stored at −80 °C prior to initiating subcellular fractionation.

Isolation of the MCF7 DNA synthesome

The DNA synthesome-containing protein fraction was purified essentially as described by Lin et al. [20] and Malkas et al. [24] and as outlined in Fig. 1. Briefly, frozen cell pellets from exponentially grown MCF7 cells were thawed and resuspended in three volumes of the homogenization buffer containing 50 m*M* HEPES (pH 7.5),

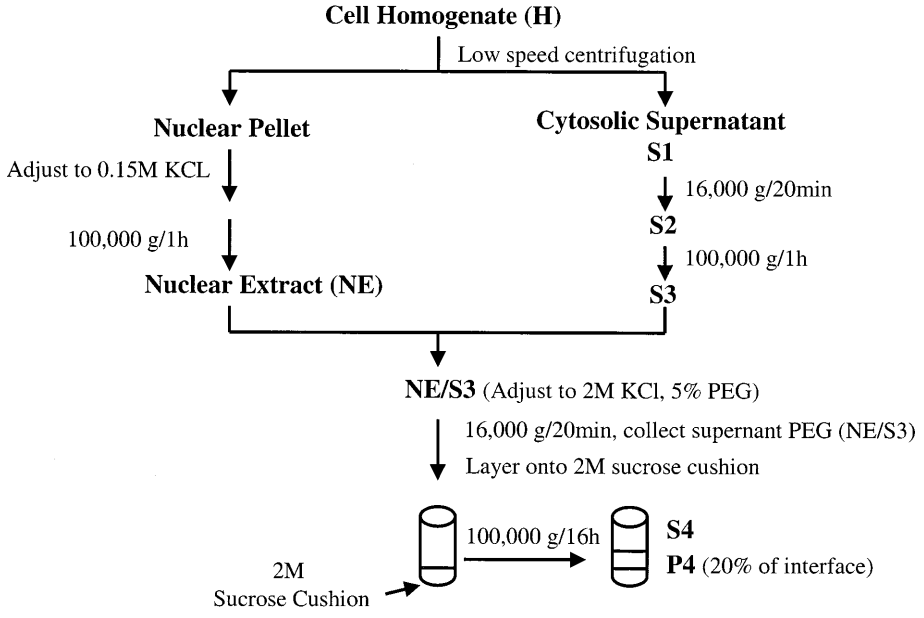


Fig. 1 Purification scheme for the MCF7 cell DNA synthesome

200 m*M* sucrose, 5 m*M* KCl, 5 m*M* $MgCl_2$, 2 m*M* DTT and 0.1 m*M* PMSF. The pellets were homogenized using 30 strokes of a loose-fitting Dounce homogenizer, and the homogenate was centrifuged at 3000 *g* for 15 min to separate the nuclear pellet and cytosolic supernatant (S1). The nuclear pellet was resuspended in two volumes of a buffer containing 50 m*M* HEPES (pH 7.5), 400 m*M* KCl, 5 m*M* $MgCl_2$, 5 m*M* EDTA, 2 m*M* DTT, and 0.1 m*M* PMSF and rocked for 2 h at 4 °C followed by centrifugation at 100,000 *g* for 1 h at 4 °C, and the supernatant (NE) was collected. EDTA and EGTA were added to a concentration of 5 m*M* to the S-1 fraction, and the S1 fraction was centrifuged at 16,000 *g* for 20 min. The supernatant (S2) was centrifuged at 100,000 *g* for 1 h to prepare the S3 fraction.

The NE and S-3 fractions were combined, and both KCl and polyethylene glycol (PEG 8000) were added to the combined NE/S3 to a final concentration of 2 *M* and 5%, respectively. The mixture was rocked for 1 h at 4 °C followed by centrifugation at 16,000 *g* for 15 min. The supernatant was dialyzed for 2 h against a buffer containing 50 m*M* HEPES (pH 7.5), 250 m*M* sucrose, 1 m*M* DTT, 150 m*M* KCl, 0.1 m*M* PMSF, and 1 m*M* each of EDTA and EGTA. The dialyzed fraction was clarified by centrifugation at 15,000 *g* for 15 min, and the resulting supernatant was layered onto a 2 *M* sucrose cushion and centrifuged at 100,000 *g* for 18 h at 4 °C. Following centrifugation, 20% of the interface of the sample above the sucrose cushion was collected and designated as P4. The upper 80% of the solution was designated the S4 fraction. The P4 fraction was dialyzed in a buffer containing 20 m*M* HEPES (pH 7.5), 5 m*M* KCl, 1.5 m*M* $MgCl_2$, and 1 m*M* DTT, centrifuged at 4 °C for 10 min at 3000 *g* and stored in aliquots at −80 °C until needed.

Measurement of intact cell DNA synthesis

Exponentially growing MCF7 cells (5×10^4) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. The cells were then exposed for 24 h to increasing concentrations of drug prior to labeling with [^{3}H]thymidine. After 4 h of incubation, the cells were lysed and the level of DNA synthesis was measured in terms of the amount of [^{3}H]thymidine retained in acid-insoluble material.

Clonogenicity assay

Cells (10^3) were seeded onto 60-mm cell culture plates and incubated for 24 h at 37 °C. The cells were then exposed to increasing concentrations of drug for 4 h. After incubation, the medium was removed and the cells were washed with PBS and then incubated for 5 days in fresh drug-free medium. Colonies were visualized by staining with Giemsa stain, and the number of colonies formed on each plate was quantified by counting the stained colonies with a diameter ≥1 mm.

DNA polymerase α assay

Using our previously published procedure [24], DNA polymerase α activity was assayed in the absence or presence of increasing concentrations of drug using an activated calf thymus DNA as the template (Sigma Co.). Briefly, 20 µl reaction mixture contains 20 µg of synthesome fraction, 20 m*M* Tris-HCl (pH 8.0), 8 m*M* $MgCl_2$, 1 m*M* DTT, 0.1 m*M* each of dATP and dGTP, 0.01 m*M* dCTP, 2 µg activated calf thymus DNA template, and 0.1 µCi [^{3}H]dTTP. The reaction was started by incubating the reaction mixture in the absence or presence of increasing concentrations of dFdCTP or araCTP for 1 h at 37 °C. The amount of [^{3}H]dTTP incorporated was quantified by liquid scintillation counting after spotting the reaction mixture on DE81 filters as described by Malkas et al. [24].

DNA polymerase δ assay

DNA polymerase δ activity was detected using a poly(dGdC) template. A 12.5-µl volume of assay mixture contained 12.5 ng of template, 5% glycerol, 2 mg bovine serum albumin, 25 m*M* HEPES (pH 5.9), 10 m*M* $MgCl_2$, 10 µ*M* dCTP, 0.25 µCi [α-^{32}P]dGTP (3000 Ci/mmol), and 5 µg synthesome protein. The reactions were carried out at 37 °C with increasing concentration of drugs for 15 min. The whole reaction mixture was spotted onto Whatman DE81 filters. The filters were then processed to quantify the amount of radiolabeled nucleotide incorporated into the DNA template [10].

In vitro SV40 replication assay

The assay was performed essentially as described previously [24, 32] in the absence or presence of increasing concentrations of drug. The reaction mixture (25 µl) contained 30 m*M* HEPES (pH 7.5), 7 m*M* $MgCl_2$, 0.5 m*M* DTT, 2 µg SV40 large T antigen, 20 µg of synthesome protein fraction, 50 ng of the plasmid $pSVO^+$

containing an inserted SV40 replication-origin DNA sequence, 1 μCi [α-^{32}P]dGTP (3000 Ci/mmol), 100 μ*M* each of dATP, and dTTP, 10 μ*M* each of dCTP and dGTP, 200 μ*M* each of rCTP, rGTP, and rUTP, 4 m*M* rATP, 40 m*M* phosphocreatine, and 1 μg creatine phosphokinase. The replication reaction was started by incubating the reaction mixture at 37 °C for 4 h. The reaction mixture was spotted onto Whatman DE81 filters and quantified by liquid scintillation counting. For gel analysis of the replication products, the reaction was stopped by adding 100 μg yeast RNA in 1% SDS followed by digestion for 1 h at 37 °C with 2 μg proteinase K. DNA replication products formed in the assay were then isolated by extracting the digestion mixture twice with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. The extracted DNA was then precipitated in the presence of 2 *M* ammonium acetate with 2-propanol and the DNA pellet collected by centrifugation and resuspended in 10 m*M* Tris/1 m*M* EDTA. The products were analyzed using either a 1% neutral agarose gel containing TBE (90 m*M* Tris/90 m*M* boric acid/2 m*M* EDTA) or a 1% alkaline gel containing 50 m*M* NaOH/1 m*M* EDTA. The gels were dried and autoradiograms of the dried gels were produced by exposing the dried gels to Kodak film at −80 °C.

Purification of the SV40 large T antigen

T antigen was purified as previously described [24, 30].

Results

Effect of dFdC and araC on intact MCF7 cell DNA synthesis

In order to verify that araC and dFdC affect the ability of intact MCF7 cells to carry out DNA synthesis as previously reported [1, 11, 16], exponentially growing MCF7 cells were incubated in the absence (control, assigned a value of 100%) or presence of increasing concentrations of araC and dFdC. The cells were exposed for 24 h to drug concentrations ranging from 0.1 to 1000 μ*M*, and the drugs were then removed by washing the cells with PBS. Fresh medium was added to the cell culture and the cells were incubated with [^{3}H]thymidine for 4 h. The labeled cells were lysed, and the level of DNA synthesis was determined by quantifying the amount of [^{3}H]thymidine retained in acid-insoluble material. As shown in Fig. 2, intact MCF7 cell DNA synthesis was inhibited by both drugs in a concentration-dependent manner. About 10 μ*M* dFdC and 80 μ*M* araC were required to reduce MCF7 cell DNA synthesis to 50% of the control activity measured in the absence of either drug. The IC_{50} value for the inhibition of intact cell DNA synthesis by araC was comparable to that observed by us using the estrogen-receptor-negative breast cancer cell line, MDA MB-468 [1]. We also observed that the araC concentration required to inhibit 50% of intact HeLa cell DNA synthesis was also about 80 μ*M* (data not shown). This value was consistent with our previously reported IC_{50} value for araC inhibition of intact cell DNA synthesis [22].

We next performed cell survival assays to compare the cytotoxic effects of dFdC and araC on the ability of MCF7 to form colonies (Fig. 3). at concentrations of dFdC above 8 μ*M* 50% of the cells lost their clonogenic capacity following a 4-h incubation with the drug. The concentration of dFdC needed to inhibit the clonogenic

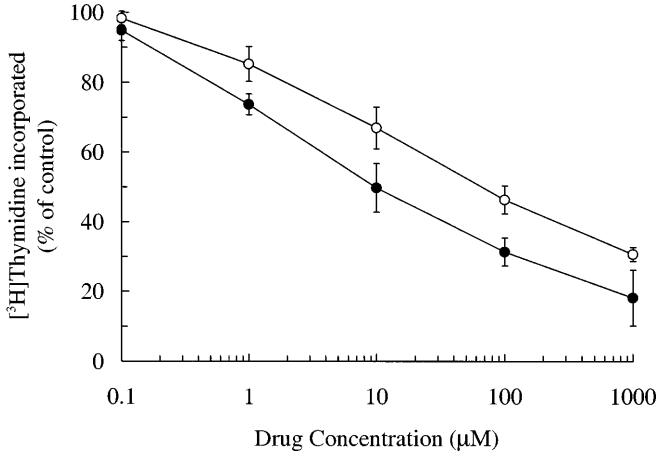


Fig. 2 Effect of dFdC and araC on intact MCF7 cell DNA synthesis. MCF7 cells (5×10^4) were seeded onto 60 mm cell culture plates and incubated for 24 h at 37 °C in Joklik's modified Eagle's medium. The cells were then exposed to one of several different concentrations of the indicated drug for 24 h at 37 °C. The cells were then labeled with [^{3}H]thymidine (1 μCi/ml of medium). After a 4-h incubation, the cells were lysed and the level of DNA synthesis was determined by quantifying the amount of [^{3}H]thymidine present in acid-insoluble material (○ araC, ● dFdC). Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Cells grown and labeled in the absence of drug served as the controls (100%) to which the drug-treated cells were compared

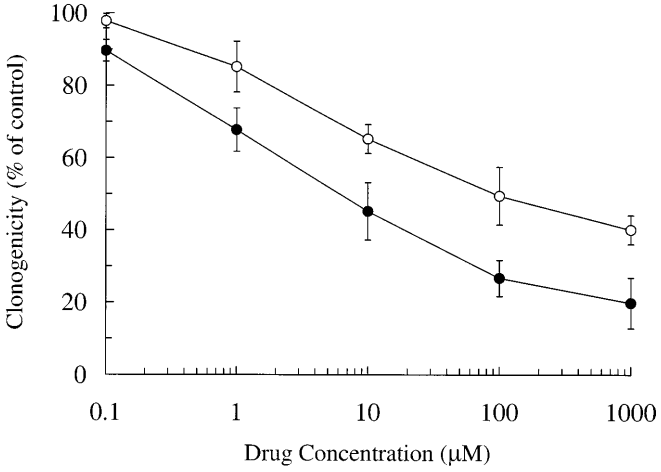


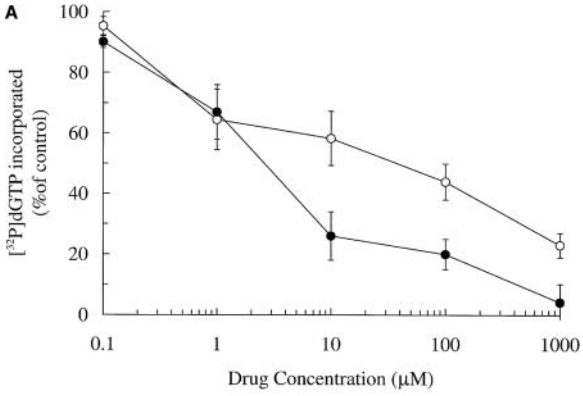
Fig. 3 Effect of dFdC and araC on the clonogenicity of intact MCF7 cells. MCF7 cells (10^3) were seeded onto 60 mm cell culture plates and incubated for 24 h at 37 °C in Joklik's modified Eagle's medium. Cells were then exposed to different concentrations of drugs for 4 h followed by incubation in drug-free medium for 5 days. Colonies were fixed with 10% formaldehyde in PBS and visualized by Giemsa stain and counted as described in Materials and methods (○ araC, ● dFdC). Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Cells grown and labeled in the absence of drug served as the controls (100%) to which the drug-treated cells were compared

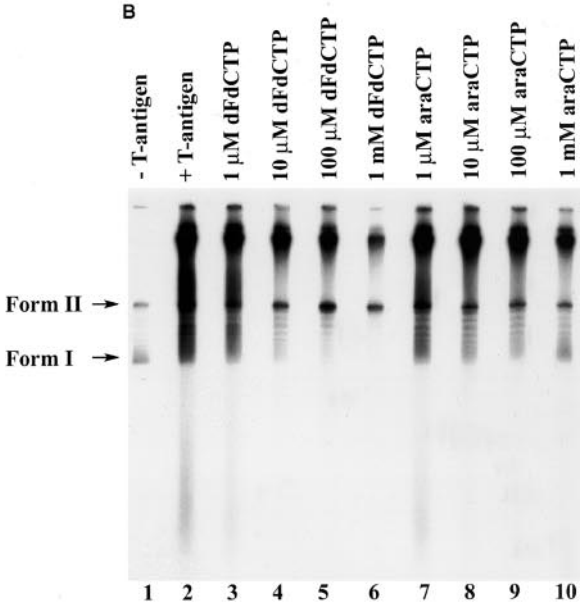
survival of 50% of MCF7 cells was approximately 12-fold less than that of araC. Thus, our results from the intact DNA synthesis and clonogenicity demonstrated that dFdC was significantly more cytotoxic to intact MCF7 cells than araC.

Comparison of the inhibitory effects of araCTP and dFdCTP on in vitro SV40 origin-dependent DNA replication using the MCF7 cell DNA synthesome

We have previously shown that the DNA synthesome isolated from the HeLa cell and human breast cancer cell MDA MB-468 is capable of supporting the origin-specific T antigen-dependent SV40 DNA replication reaction in vitro [1, 4, 32]. These studies demonstrated the utility of the purified DNA synthesome as a relevant in vitro model that is useful for studying the mechanism of action of anticancer drugs such as araC, camptothecin, and VP16. In order to directly compare the anti-DNA synthetic activity of araCTP and dFdCTP, we performed in vitro SV40 origin-dependent DNA replication assays in the absence and presence of several concentrations of each of these two drugs. Both drugs inhibited SV40 origin-containing DNA replication in a concentration-dependent manner as measured by quantifying the amount of [^{32}P]dGTP incorporated into DNA (Fig. 4A). A 50% inhibition of the in vitro DNA replication assay was achieved in presence of 10 μ*M* dCTP using approximately 3 μ*M* dFdCTP and 60 μ*M* araCTP. The results of this assay indicated that dFdCTP was able to more effectively compete with dCTP to inhibit synthesome-mediated in vitro DNA replication than araCTP. Our data showed a close correlation between the IC_{50} values of both drugs for inhibiting intact cell DNA synthesis and the DNA synthesome-mediated in vitro SV40 replication assay.

We further analyzed the replication products of the in vitro replication reaction using a 1% neutral and a 1% alkaline agarose gels (Fig. 4B,C). Our results indicated that the MCF7 cell DNA synthesome was capable of ▶





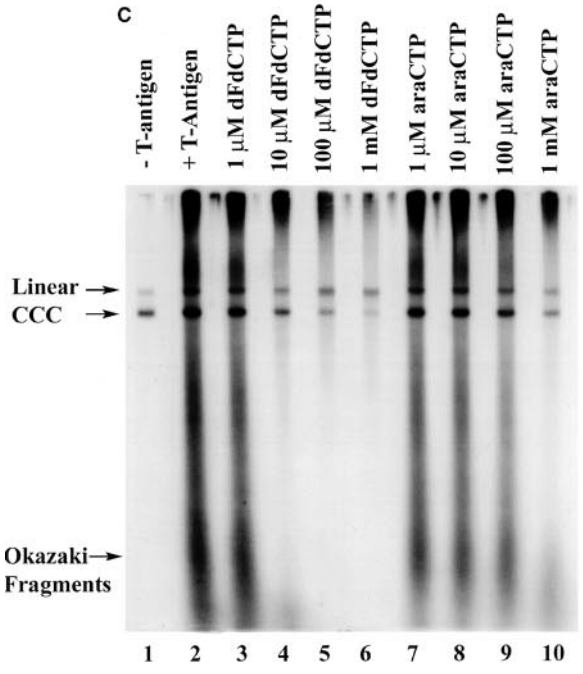


Fig. 4A–C Effect of dFdCTP and araCTP on DNA synthesome-mediated in vitro SV40 DNA replication. **A** Inhibition of dFdCTP and araCTP on the synthesome-mediated in vitro SV40 DNA synthesis as a function of drug concentrations (○ araCTP, ● dFdCTP). The assays were performed as described in Materials and methods. Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Control reactions were performed in the absence of drug (100%). **B** Neutral agarose gel analysis of the reaction products of the in vitro SV40 DNA replication assay. **C** Alkaline agarose gel analysis of the reaction products of the in vitro SV40 DNA replication reaction. The DNA replication products formed in the in vitro DNA replication reaction were isolated by phenol/chloroform extraction followed by precipitation at room temperature with 2-propanol in the presence of 2 *M* ammonium acetate. The isolated DNA was resuspended in 10 m*M* Tris/1 m*M* EDTA, and the reaction products were resolved using 1% agarose gels under either neutral or alkaline conditions (Materials and methods). The gels were dried and exposed to Kodak XAR5 films at −80 °C for 8 h (*CCC* covalently closed circular DNA)

producing full-length daughter DNA as shown by the presence of form I (superhelical) DNA and form II (nicked open circular) DNA, as well as higher-order replication intermediates (Fig. 4B, lane 2). This reaction was also T antigen-dependent (Fig. 4B, compare lanes 1 and 2). In the presence of low concentrations (1 μ*M*) of both drugs, full-length daughter DNA molecules (form I and form II) were observed (Fig. 4B, lanes 3 and 7). At higher concentrations, the production of form II DNA molecules as well as replication intermediates was inhibited in a concentration-dependent manner (note both neutral and alkaline gels, Fig. 4B,C, lanes 2–10). However, form I DNA molecules disappeared at drug concentrations higher than 1 μ*M*, indicating that dFdCTP and araCTP may impair the process to form supercoiled DNA. In the presence of both drugs, the production of short Okazaki fragments was inhibited in a concentration-dependent manner (Fig. 4C, lanes 3–10) and was completely inhibited at higher dFdCTP concentrations (Fig. 4C, lanes 5 and 6), suggesting that dFdCTP had a greater inhibitory effect on the initiation stage of DNA synthesis.

These results are in accordance with our previously reported results using the DNA synthesome isolated from HeLa cells and MDA MB-468 cells [1, 22]. Furthermore, they correlate with the results of other studies carried out in this laboratory employing our DNA synthesome-mediated in vitro DNA replication assay system as well as intact cells [16, 28].

The effects of araCTP and dFdCTP on DNA synthesome-associated DNA polymerase α and DNA polymerase δ activity

Our previous study on the inhibitory effects of araCTP on the activity of the purified DNA polymerase α and the DNA synthesome-associated polymerase α provided evidence indicating that the DNA synthesome can be used as an in vitro model system that more closely reflects the events occurring within the intact cell than can be achieved using individually purified enzymes [32, 33].

To further compare the activities of araC and dFdC on individual DNA replication essential proteins, we performed DNA synthesome-associated DNA polymerase α and δ assays. In the polymerase α assays, we used activated calf thymus DNA as the template, and incubated the template with various concentrations of araCTP and dFdCTP in the presence of the DNA synthesome (Materials and methods). The polymerase α activity was determined by quantifying the amount of [^{3}H]TTP incorporated into DNA (Fig. 5). Unlike in vitro SV40 DNA replication, the activity of the synthesome-associated polymerase α was not inhibited by 1 μ*M* of either drug. However, at approximately 80 μ*M* dFdCTP and 100 μ*M* araCTP the activity of the synthesome-associated DNA polymerase was inhibited by 50% relative to the activity of the control reaction performed in the absence of the drug.

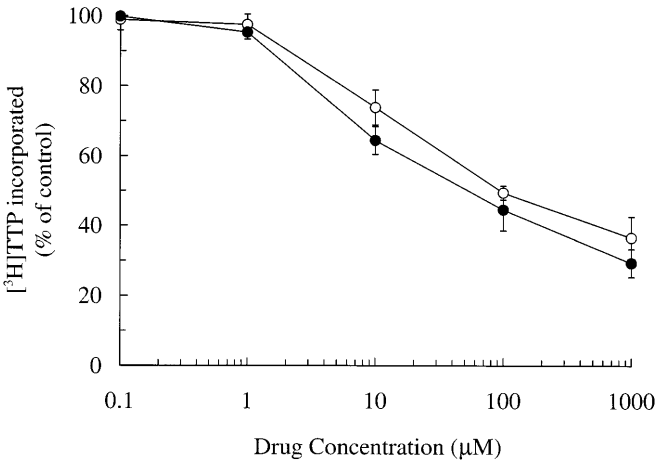


Fig. 5 Effect of dFdCTP and araCTP on DNA synthesome-associated DNA polymerase α activity. The assays were performed using activated calf thymus DNA as the template. Reaction mixtures were incubated with different concentrations of drugs as described in Materials and methods (○ araCTP, ● dFdCTP). The amount of [^{3}H]dTTP incorporated into DNA was determined in terms of binding to Whatman DE81 filters [21]. Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Control reactions were performed in the absence of drug (100%)

Our results indicate that the inhibitory effect of dFdCTP and araCTP on synthesome-associated DNA polymerase α occurs at nearly equivalent concentrations. The IC_{50} value of araC was in good agreement with that previously reported [1, 10, 32]. However, dFdCTP was not significantly more potent than araCTP in the DNA polymerase assay, and this contrasts with our results obtained using the in vitro SV40 DNA replication assay.

We have previously reported that the inhibitory effect of araCTP is primarily through inhibition of synthesome-associated DNA polymerase α and that a significant amount of inhibition of synthesome-associated polymerase δ activity also occurs, but at a fourfold higher concentration of the drug [1, 10]. In this study, it was also observed that both dFdCTP and araCTP showed inhibition of DNA polymerase δ at higher concentrations than those required to inhibit DNA polymerase α. The IC_{50} values of dFdCTP and araCTP for inhibition of DNA polymerase δ were approximately 700 μ*M* and 750 μ*M*, respectively (Fig. 6). However, the activity of synthesome-associated polymerase δ was not significantly inhibited by either drug at a concentration of 100 μ*M*. This was a significant finding because the activity of DNA polymerase α was readily inhibited by apparently seven- to ninefold less drug than DNA polymerase δ.

The fact that dFdCTP was significantly more potent than araCTP at inhibiting in vitro SV40 replication than at inhibiting synthesome-associated DNA polymerase α and δ activity strongly suggests that dFdCTP may have a greater effect on inhibiting the coordinated replication activity of an organized DNA replication multienzyme complex than purified DNA polymerases. Furthermore,

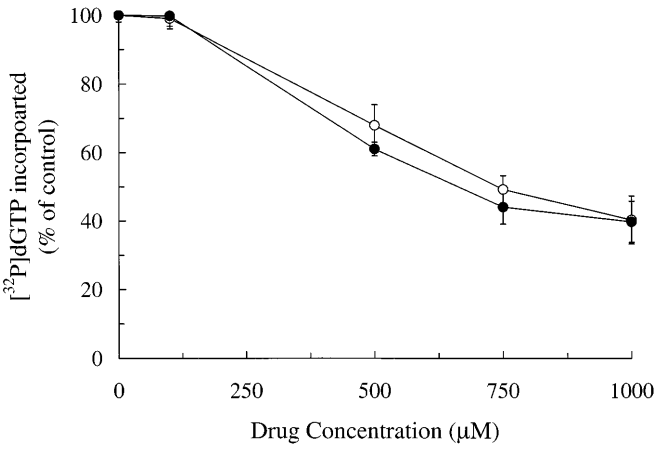


Fig. 6 Effect of dFdCTP and araCTP on the synthesome-associated DNA polymerase δ activity. The assay was performed using a poly(dGdC) template as described in Materials and methods (○ araCTP, ● dFdCTP). The amount of [α-^{32}P]dGTP incorporated into DNA was determined in terms of binding to Whatman DE81 filters [9]. Each point represents the average of three separate experiments; error bars represent the standard errors of the means. Control reactions were performed in the absence of drug (100%)

our results imply that the potent inhibitory effects of dFdC may be due to the drug targeting additional proteins during the DNA synthetic process, or inhibiting the coordinated elongation of both strands at a replication fork by specifically slowing the ability of one of the polymerases at the replication fork to efficiently carry out DNA synthesis.

Discussion

dFdC is a novel deoxycytidine analog with both structural and metabolic similarities to araC. AraC is one of the most effective drugs available today for the treatment of acute leukemia and other hematopoietic malignancies. dFdC is also effective against leukemia [14, 21], and has also proven to be effective against a variety of solid tumors. Like araCTP, dFdCTP inhibits DNA synthesis mainly through the inhibition of the activity of the DNA polymerases. A considerable body of work has accumulated on the effects of these two drugs on intact cell DNA synthesis in a variety of cancer cell lines. Using a cell survival assay, Heinemann et al. [11, 12] have discovered that Chinese hamster ovary cells are significantly more sensitive to dFdC than to araC after both a 4- and an 18-h incubation. Our study using the human breast cancer cell line MCF7 in a clonogenic assay also showed that dFdC is approximately 15-fold more cytotoxic than araC following a 24-h incubation with the drug. dFdC inhibits [^{3}H]thymidine uptake approximately tenfold more than araC in intact MCF7 cells. Accumulation of dFdCTP has been observed to be cell line-dependent, with the cell lines that are more sensitive to the drug accumulating higher amounts of dFdCTP in cultures [16, 25]. This may explain the difference between the IC_{50} values obtained in our experiments, and those obtained by other investigators who have performed similar studies with CHO cells [11], human T-lymphoblastoid CCRF-CEM cells [15] and HL60 cells [29].

DNA synthesis is the most prominent activity inhibited by dFdC in cultured cells [11]. Huang et al. [16] have directly investigated the molecular mechanism of action of dFdC and araC in vitro on DNA synthesis using purified DNA polymerase α and ε. However, the use of purified DNA polymerases may not adequately reflect the DNA synthetic process as it occurs within the intact cell. In the intact cell, DNA synthesis involves the coordinated activity of DNA polymerases α and δ along with that of several other enzymes and factors. In this report, we describe studies performed with intact MCF7 cells and the DNA synthesome isolated from these cells. We directly compared the effectiveness of dFdCTP and araCTP as inhibitors of the DNA synthetic process. dFdCTP was significantly more potent than araCTP in the in vitro SV40 DNA replication assay. Full-length DNA was produced in the presence of very low levels of both drugs, suggesting that incorporation of dFdCTP and araCTP did not stop the polymerases from elongating the DNA template.

Our results are in agreement with those of Ross et al. [29], who have demonstrated that dFdC is progressively incorporated into nascent DNA of increasing size in intact HL60 cells. In contrast, studies of dFdCTP incorporation using in vitro primer extension assays by purified DNA polymerase α and δ have demonstrated that after incorporation of dFdCTP to the 3′ terminus of the elongating DNA strand, one more deoxynucleotide can be added before the DNA polymerases are unable to continue elongating the nascent strand. Therefore, dFdC appears to act as a chain terminator [16]. Although dFdCTP appears to be a more potent inhibitor of SV40 DNA synthesis in vitro than araC, the inhibitory effects of dFdCTP and araCTP on the activity of the DNA synthesome-associated DNA polymerase α and δ are similar. The IC_{50} values of dFdCTP and araCTP for DNA polymerase α are 80 μM and 100 μM, respectively. These values are comparable to the intracellular dFdCTP concentration, which has been reported to range from 64 μM to 362 μM in the leukemia cells of patients undergoing dFdCTP therapy [7, 8].

Taken together, our results and those reported by others [6, 7] suggest that DNA polymerase α is a major target for dFdCTP and araC. Inhibition of DNA synthesome-associated polymerase δ occurred, but at substantially higher drug concentrations than required to inhibit DNA polymerase α to the same extent. About 300 μM of dFdCTP was required to inhibit DNA polymerase δ by 10%. Therefore, it is most likely that the inhibitory effect of dFdCTP and araCTP on the SV40 origin-dependent DNA replication process is mediated primarily through inhibition of DNA polymerase α. This conclusion agrees with the findings of our previous studies analyzing the mechanism of action of araCTP in this same assay [1, 10, 32, 33].

The fact that dFdCTP was significantly more potent than araCTP suggests that dFdCTP may have more impact on the organized multiprotein DNA replication complex (i.e. the DNA synthesome) found in intact cells than would be suggested from studies employing purified individual DNA polymerases. Furthermore, studies from intact cells and in vitro assays have shown that addition of dCTP cannot completely restore DNA synthesis to the level observed in assays performed without dFdCTP [16]. This observation indicates that the inhibition of DNA polymerase α activity by dFdC is not simply a result of competition with dCTP. Primer extension assays have shown that the $3' \rightarrow 5'$ exonuclease activity of purified DNA polymerase ε is essentially unable to excise nucleotide from DNA containing dFdCMP at either the 3′ terminus or from an internal position within the DNA. AraCMP, however, has been reported to be removed from the 3′ terminus of DNA [16], indicating that dFdC may have more impact on DNA repair than previously thought.

However, the greater anticancer activity of dFdC is not only attributed to the inhibition of DNA polymerase α activity, but may be derived from damage to the DNA into which dFdC is incorporated. dFdC is more potent than araC for several reasons. First, the chemical addition of fluorine atom to the 2′ position of the furanose ring of the drug makes dFdC more lipophilic and therefore more permeable to cells than araC. Second, deoxycytidine kinase has a higher affinity for dFdC than araC which leads to higher levels of dFdCTP than araCTP in cells. Third, dFdC inhibits ribonucleotide reductase which causes depletion of the cellular pools of deoxynucleoside triphosphate, particularly the levels of the competing metabolite, dCTP. Thus, the ratio of cellular dCTP to dFdCTP favors the inhibition of DNA synthesis by dFdCTP [6, 12]. In contrast, araC has no effects on dNTP pools and is not known to act as an inhibitor of ribonucleotide reductase which helps maintain higher levels of active metabolite within the cell.

It has been reported that the cellular dCTP level is 3.5 μM in K562 cells [5]. Since the concentration of dCTP used in our in vitro assays was 10 μM, it is possible that the differential effects of dFdC and araC on DNA synthesis might be more dramatic if true intracellular dCTP concentrations were used. Finally, the elimination of cellular dFdCTP is slower than that of araCTP [16]. All of these characteristics of dFdCTP contribute to the higher intracellular concentration of dFdCTP as compared to araCTP. dFdCTP is found at from 9- to 20-fold higher concentrations in treated cells than in cells treated with equivalent concentrations of araC [11]. However, the increase in the intracellular concentration of dFdCTP does not completely account for the greater cytotoxicity of dFdCTP in intact cells, which has been reported to be 180-fold more toxic than araC [12, 16, 26, 27].

In conclusion, in this study we demonstrated that dFdCTP is significantly more potent than araCTP at inhibiting the DNA synthetic process in our cell-free SV40 origin-dependent in vitro DNA replication assay system employing the human MCF7 cell DNA synthesome. The results of this study indicate that the DNA synthesome can serve as a relevant in vitro model system for studying the mechanism of action of anticancer drugs that directly affect DNA synthesis, and that the mechanisms through which these drugs inhibit in vitro DNA synthesis closely parallel the inhibitory effects of these drugs in intact cells [1, 4, 10, 32, 33]. We have demonstrated in our laboratory that the DNA synthesome is able to incorporate araCTP into internucleotide linkages, and that this incorporation of araC into internucleotide linkages more closely resembles the molecular events occurring in intact cells than can be achieved using purified DNA polymerases [32, 33].

Continued analysis of the mechanisms by which dFdCTP mediates its cytotoxic effects will uncover the effects dFdC has on the initiation, elongation and termination stages of the DNA synthesis process. Thus, our in vitro model system, which utilizes the DNA synthesome to mediate the DNA synthetic reaction is anticipated to be of substantial value for gaining insight into the mechanism(s) of action of dFdCTP and other anticancer drugs that directly inhibit cellular DNA synthesis. Continued validation of the in vitro DNA replication model system employing the DNA synthesome is therefore expected to show the system to be of considerably more value in the search for more effective anticancer drugs than conventional model systems that only employ highly purified individual enzymes such as DNA polymerase or topoisomerase II.

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