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Original Article

DNA lesion bypass polymerases and 4'-thio-β-D-arabinofuranosylcytosine (T-araC)

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Abstract: The 4'-thio-β-D-arabinofuranosylcytosine (T-araC) is a newly developed nucleoside analog that has shown promising activity against a broad spectrum of human solid tumors in both cellular and xenograft mice models. T-araC shares similar structure with another anticancer deoxycytidine analog, β-D-arabinofuranosylcytosine (araC, cytarabine), which has been used in clinics for the treatment of acute myelogenous leukemia but has a very limited efficacy against solid tumors. T-araC exerts its anticancer activity mainly by inhibiting replicative DNA polymerases from further extension after its incorporation into DNA. DNA lesion bypass polymerases can manage the DNA lesions introduced by therapeutic agents, such as cisplatin and araC, therefore reduce the activity of these compounds. In this study, the potential relationships between the lesion bypass Y-family DNA polymerases η, ι and κ (pol η, pol ι, and pol κ) and T-araC were examined. Biochemical studies indicated that the triphosphate metabolite of T-araC is a less preferred substrate for the Y-family polymerases. In addition, cell viability study indicated that pol η deficient human fibroblast cells were more sensitive to T-araC when compared with the normal human fibroblast cells. Together, these results suggest that bypass polymerases reduced cell sensitivity to T-araC through helping cells to overcome the DNA damages introduced by T-araC.

Keywords: Bypass polymerases, nucleoside analogs, araC

Introduction

Antimetabolite nucleoside analogs are an important class of compounds for anticancer chemotherapy. For example, fluorouracil (5-FU, fluoroplex), a pyrimidine analog, has been used for treating cancer for 50 years. In addition, deoxycytidine analogs cytarabine (β-D-arabinofuranosylcytosine, araC) has been used for treating AML [1] and gemcitabine (β-D-2′,2′-difluorodeoxycytidine, dFdC) has been used for treating various types of cancers, including non small cell lung and pancreatic cancer [2, 3]. Although araC is effective in treating hematological malignancies, it is not effective against solid tumors [4]. One of a newly developed anticancer nucleoside analogs, 4'-thio-β-D-arabinofuranosylcytosine (T-araC, **Figure 1**), has a structure similar to araC except the 4'-oxygen atom was replaced with a sulfur (**Figure 1**). Interestingly, T-araC exhibits an improved activity against a variety of human solid tumor in xenograft models as compared to araC and gemcitabine [5, 6].

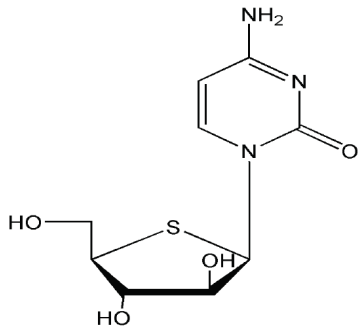

Figure 1. The structure of T-araC.

Inhibition of DNA polymerases is the key mechanism of action for both araC and gemcitabine. T-

araC shares the similar mechanism of action with araC and gemcitabine. T-araC can also be phosphorylated into its mono-, di-, and triphosphate metabolites by cellular kinases such as deoxycytidine kinase [7]. The triphosphate metabolite of T-araC is a substrate for cellular replicative DNA polymerases [7-10]. Furthermore, the triphosphate metabolite (T-araCTP) has a half-life 5 to 10 times as long as that of araCTP [5], which implicates a better opportunity to be incorporated into DNA by cellular polymerases. Although there is a 3' hydroxyl group on the sugar moiety of both araC and gemcitabine, it has been shown that the incorporated araC or gemcitabine at the 3' termini of DNA inhibits further extension and therefore prematurely terminates DNA elongation process [11]. The incorporated T-araCMP at the 3' termini of DNA also has a strong inhibitory effect on DNA polymerase for further extension [10]. Therefore, cellular DNA polymerases with the ability to incorporate T-araC into the genomic DNA play key roles in determining the cytotoxic effect of T-araC.

In addition to the replicative DNA polymerases, which are responsible for replicating the genome during cell duplication, specialized DNA lesion bypass (TLS) DNA polymerases that help cells to overcome damaged DNA have been discovered and play important roles in maintaining genome stability [12]. For example, DNA polymerase η (pol η) was discovered to have the ability to replicate across the UV irradiation introduced cyclobutane pyrimidine dimers (CPD) in the DNA [13], and mutations in the pol η encoding gene (Rad30A) result in a disease called Xeroderma Pigmentosum variant (XP-V) [13]. XP-V patients are highly sensitive to sunlight and prone to skin cancer development in sun-exposed areas [14]. Furthermore, cells derived from XP-V patients have higher mutation rates [15]. In addition to pol η, polymerases ι, κ and ζ were also discovered and belong to the new Y-family DNA polymerases [16]. In contrast to the replicative DNA polymerases, Y-family DNA polymerases have relative low efficiency, low fidelity but the ability to replicate across various DNA lesions that impede the replicative polymerases during replication processes [16]. For example, pol ι, encodes by human Rad30B gene and paralog of pol η, incorporates dGMP opposite thymidine 3-10 times more frequently than the correct nucleotide dAMP [17, 18]. Pol ι can bypass the oxidative DNA lesion 8-oxoguanine and has been demonstrated to participated in the DNA base excision processes [19]. DNA polymerase kappa (pol κ) is another Y-family polymerase member that has an error rate of 1 in every 10^2 to 10^3 nucleotides incorporation events [20]. Human Pol κ bypasses many N2-adducted dG residues efficiently and accurately, and pol κ is considered to be specialized in its ability to bypass N2-adducted dG lesions [21-23]. Pol κ bypasses N2-furfuryl-dG and N2-(1-carboxyethyl)-2-dG residues with high accuracy and increased efficiency opposite relative to an undamaged dG [24]. Pol κ also bypasses benzo[a]pyrene adduct in DNA and loss of pol κ sensitizes cells to the killing by benzo[a]pyrene and increases mutagenesis induced by benzo[a]pyrene [25]. In addition, loss of pol κ also increases cell sensitivity to DNA-alkylating agents and to UV irradiation [26]. Pol κ also has the ability to extend mismatched primer termini, which suggests it's potential function as an "extender" polymerase when two TLS polymerases are required for bypassing DNA lesions [27].

While these TLS polymerases provide cells an alternative pathway to handle DNA lesions during DNA replication, studies have also shown that these bypass polymerases affect the cytotoxicity of nucleoside analogs and other therapeutic agents [28, 29]. For example, DNA polymerase η (pol η), one of the TLS polymerases, was shown to be able to extend DNA with either araC or gemcitabine at the 3' termini and bypass the araC or gemcitabine sites in template DNA [28]. These activities of pol η result in a reduced cellular sensitivity to both araC and gemcitabine [28].

T-araC is a newly developed nucleoside analog and its potential relationship with lesion bypass polymerases is not understood to date. In this study, we examined the interactions between T-araC and the Y-family DNA polymerases η, ι, κ, at both biochemical and cellular levels.

Materials and methods

Enzymes and DNA oligonucleotides

T-araCTP was kindly provided by Dr. William Parker (Southern Research Institute, Birmingham, AL) and araCTP was synthesized as previously described [28]. Human pol η and ι were overexpressed and purified as described [28]. Human DNA polymerase κ was a generous gift from Dr. Errol Freidberg (UT, Southwestern).

DNA oligonucleotide primer was labeled with fluorophore Quasar 570 at the 5′ ends and annealed to a template oligonucleotide to form recessed DNA substrates for extension studies. The recessed dsDNA substrates are: 5′-GTGGCGCGGAGACTTAGAGC-3′ and 3′-CACCG CGCCTCTGAATCTCGGTAAACCGCGCCCCTTAAGG -5′.

Enzymatic reactions

Standard polymerase reactions containing 100 nM primer-template DNA, 300 μM nucleoside analogue triphosphates, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL bovine serum albumin, and 1 nM DNA polymerase were incubated at 37°C for 5 minutes in 10 μL. The reactions were stopped by adding formamide and heating at 95°C for 5 minutes. The reaction mixtures were loaded on a 12.5% denaturing gel for electrophoresis.

Cell culture and viability assays

SV40-transformed human fibroblast cells that exhibited normal (GM637) or pol η-deficient (XP30RO) have been described [28]. Exponentially growing cells were seeded in 24-well plates for 24 hours before the addition of drug in triplicates. The cells were treated with drugs for 72-hour at 37°C. At the end of the time course, 5 mg/mL MTT (100 μL) was added and incubated for 4 hours before determining the absorbance at 595 nm using a microtiter plate reader (Biorad).

Confocal microscopy

Exponentially growing XP30RO EGFP-pol η expressing cells were plated in chamber slides for 24 hours at 37°C before the treatment of T-araC (2 and 10 μM) or UV irradiation (11.3 $J{\cdot}m^{-2}$). The cells were incubated for additional 4 hours and the intracellular location of pol η was analyzed with a Leica laser scanning confocal system equipped with krypton/argon laser.

Results

Incorporation of T-araC triphosphate by DNA lesion bypass polymerases

T-araC exerts its activity mainly by terminating DNA elongation process after its incorporation into the 3' termini of DNA by cellular polymerases [9, 30]. To examine whether T-araCTP is a substrate for DNA bypass polymerases, the purified Y-family polymerases η, ι and κ were evaluated in a primer extension assay containing a recessed DNA and dCTP or T-araCTP. As shown in **Figure 2**, pol η efficiently incorporated regular dCTP, as the extension product is observed (**Figure 2**, Lane 2). In addition, pol η consecutively incorporated a second dCTP (**Figure 2**, Lane 2), in spite of a T in the template. In contrast, a reduced activity was observed when T-araCTP was used as a substrate since only trace amount of incorporation product was detected (**Figure 2**, Lane 3). Similar to pol η, pol ι also showed limited activity in incorporating T-araCTP into the DNA (**Figure 2**, lane 5). Pol κ showed a better incorporation efficiency on T-araCTP in comparison to pol η, ι, as more of the recessed DNA substrate was consumed (**Figure 2**, Lane 7). However, less incorporation products were observed as compared to that of regular dCTP (**Figure 2**, Lane 6), indicating T-araCTP is a less preferred substrate.

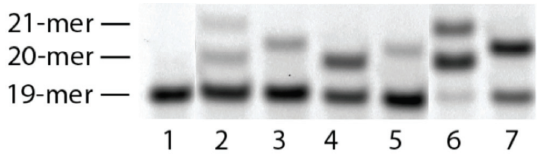

Figure 2. The incorporation of T-araCTP by DNA polymerase η, ι and κ. A recessed DNA substrate was incubated with DNA polymerase η,ι and κ in the presence of dCTP or T-araCTP. Lane 1: recessed DNA substrate. Lane 2: pol η with dCTP. Lane 3: pol η with T-araCTP. Lane 4: Pol ι with dCTP. Lane 5: Pol ι with T-araCTP. Lane 6: Pol κ with dCTP. Lane 7: pol κ with T-araCTP.

To further understand mechanisms that could contribute to the reduced incorporation efficiency of T-araC, the incorporation kinetic parameters were examined. It was difficult to obtain consistent measurement for kinetic analysis with η and ι since both enzymes showed low activity on T-araCTP incorporation reactions (**Figure 2**). The incorporation kinetic parameters of pol κ on dCTP, araCTP, and T-araCTP were determined. As shown in **Table 1**, the V_{max} of pol κ for incorporating dCTP was 1.7 fmol/min. Similar V_{max} values were observed for araCTP and T-araCTP, they were 1.9 and 1.7 fmol/min, respectively. The K_m value of pol κ on incorpo-

Table 1. The kinetic parameters of pol κ

	dCTP	araCTP	T-araCTP
V_{max} (mmol/min)	1.7	1.9	1.9
K_m (μM)	4	38	160
V_{max} /K_m	0.4	0.05	0.01

rating regular dCTP was 4 μM. In comparison, increased K_m values 38 and 160 μM were observed with araC and T-araCTP, they were 38 and 160 μM, respectively. As a result, the overall incorporation efficiency (V_{max}/K_m) of T-araC was reduced about 40-fold as compared to that of dCTP.

Cytotoxicity of T-araC in normal human and pol η deficient fibroblast cells

Although T-araC inhibits further extension reaction after its incorporation, interestingly, more internalized T-araC within DNA was found as compared to the T-araC at the DNA 3' termini in cells treated with T-araC [31], suggesting there are mechanisms (enzymes) that can internalize T-araC into DNA in cells. Previously we have shown that the expression of pol η affects the cell sensitivity to both araC and gemcitabine [28]. Biochemical analysis indicated that, unlike replicative polymerases which are inhibited by the presence of araC or gemcitabine, pol η has the ability to extend DNA with araC or gemcitabine at the 3' termini or bypass the araC or gemcitabine sites in the template DNA [28]. Since both araC and gemcitabine exert their cytotoxic activity mainly by terminating the DNA elongation processes, such activity of pol η reduces the cytotoxic effects of these compounds [28]. Since T-araC shares similar structure and mechanism of action with araC, we performed a cell sensitivity study to examine the potential impact of pol η on the cytotoxic activity of T-araC. The human fibroblast cells derived from normal and pol η deficient XP-V patient were used for the growth inhibition test [28]. As shown in **Figure 3**, the XP-V cells were more sensitive to T-araC as compared to the normal fibroblast cells. Similar relationships were observed between pol η and araC or gemcitabine [28]. These results suggest that, instead of the incorporation reaction, pol η is potentially involved in the extension or bypass reactions.

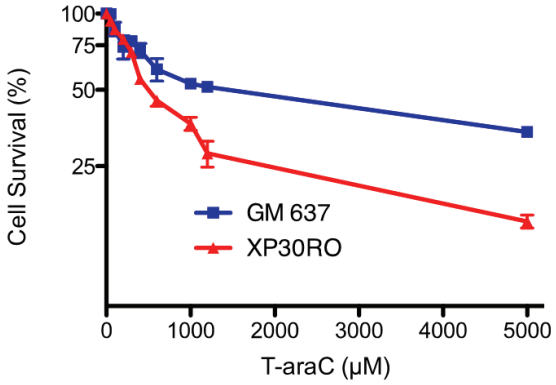

Figure 3. The cell sensitivity against T-araC. The cell sensitivity of human fibroblast cells that derived from normal (GM637) or XP-V patient (XP30RO) were determined using MTT based cytotoxicity assay. The cell survival was presented as a percentage of treated/untreated cells.

Intracellular location of pol η in response to T-araC treatment

Pol η relocates to UV irradiation induced CPD sites in cell nucleus to help replicative machinery to overcome CPD sites [32]. The intracellular relocation of pol η in response to UV irradiation is very important for pol η activity in cells. Mutant pol η that fails to relocate to CPD sites cannot complement pol η function in XP-V cells [32]. We previously have shown that the treatment of araC, gemcitabine, or other anticancer compounds such as cisplatin also induce the intracellular relocation of pol η [28]. Since pol η expressing cells are less sensitive to T-araC and T-araC prevents further DNA extension after its incorporation, it is possible that T-araC also induced pol η relocation to lesion sites to help replicative polymerase to resume the DNA replication process. As shown in **Figure 4**, treatments of T-araC also induced the intracellular relocalization of pol η to form foci in cell nucleus, similar to what were observed with araC treatment [28].

Discussion

As the current biochemical studies indicated, T-araC is a less favorable substrate for the bypass polymerases η, ι, and κ, in comparison to dCTP. The kinetic analysis of pol κ on T-araCTP incorporation reaction indicated that the reduced incorporation efficiency was mainly due to an

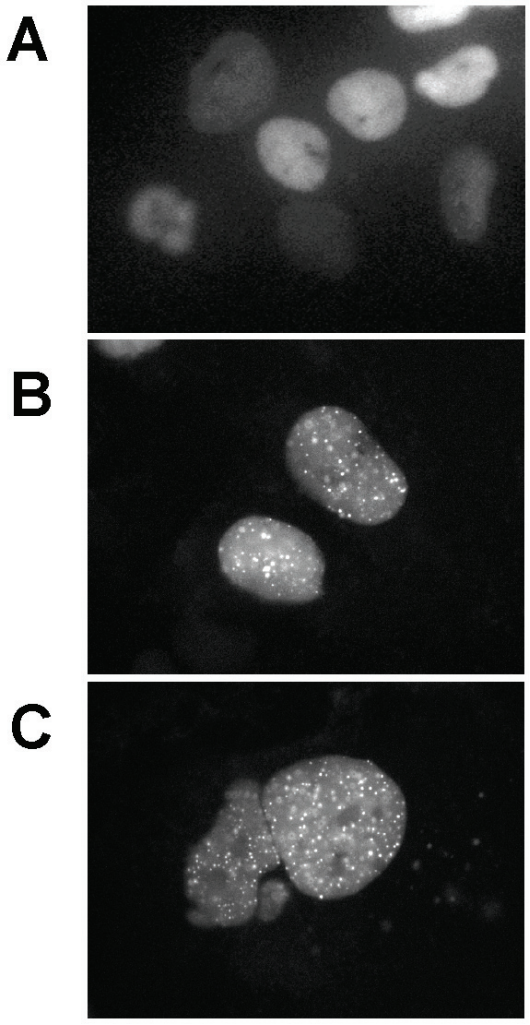

Figure 4. The intracellular location of pol η in response to T-araC treatment. A. Control, B. in the presence of 2 μM of T-araC, C. in the presence of 10 μM of T-araC.

increased K_m value as compared to dCTP (~10-fold). Although the intracellular availability of T-araCTP is longer than that of araCTP, however, the current biochemical studies suggested that these Y-family polymerases may not participate in incorporating T-araC into DNA. In addition, the cellular activity of lesion bypass polymerases is tightly regulated in the absence of damaged DNA, which also reduces the potential involvement of the bypass polymerases in incorporating T-araCTP into DNA.

The cytotoxic results indicated that the pol η expressing GM637 cells were less sensitive to T-araC than the pol η deficient cell XP30RO, which further supported that pol η is not involved in incorporating T-araC into DNA during regular DNA replication. Previously we have shown that pol η extends DNA with araC or gemcitabine at the 3' termini [28]. In addition, pol η bypasses araC or gemcitabine sites in the template DNA and the expression of pol η reduces the cellular sensitivity to both araC and gemcitabine [28]. The reduced cell sensitivity to T-araC in the pol η expressing cells maybe also contributed by the extending and bypassing ability of pol η. Our current intracellular studies also indicated that pol η relocated to form foci in response to T-araC treatment, which suggested that pol η is recruited to the T-araC induced lesion sites to help the resumption of DNA replication processes instead of in the incorporation processes.

T-araC is a newly developed nucleoside analog with promising activity against solid tumors in the xenograft model [31, 33, 34]. Similar to the mechanisms of action of araC or gemcitabine, T-araC exerts its anticancer activity mainly by terminating DNA elongation after its incorporation into DNA [9, 30]. However, previous studies have shown that the incorporation of T-araC may have limited effect on DNA synthesis during S-phase and the majority of T-araC is present in internucleotide linkage [31], our current results provided an explanation for the observed larger amount of internalized T-araC in the DNA molecules than at the 3' termini. In addition, the action of these bypass polymerases may provide the cells an alternative path to maintain DNA replication process in the presence of T-araC in the DNA. Such action of the bypass polymerases can lead to delay of the immediate apoptosis response during S-phase and lead to a G2/M arrest as previously reported [31]. Further investigation between these bypass polymerases and T-araC induced DNA damage responses are necessary to extend our understanding the mechanism of action of T-araC.

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