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Apurinic/Apyrimidinic Endonuclease-1 Protein Level Is Associated with the Cytotoxicity of L-Configuration Deoxycytidine Analogs (Troxacitabine and β-L-2′,3′-Dideoxy-2′,3′-didehydro-5-fluorocytidine) but Not D-Configuration Deoxycytidine Analogs (Gemcitabine and β-D-Arabinofuranosylcytosine)[S]

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ABSTRACT

β-L-Dioxolane-cytidine (L-OddC, BCH-4556, Troxacitabine), a novel L-configuration deoxycytidine analog, is under phase III clinical trial for cancer treatment. We showed that human apurinic/apyrimidinic endonuclease (APE-1) has exonuclease activity for preferentially removing L-OddC and other L-configuration nucleosides over D-configuration nucleosides from the 3′ terminus of DNA in vitro. In this study, we examined whether APE-1 protein plays a role in the cytotoxicity of L-OddC. We established RKO (human colorectal carcinoma) cell lines that can be induced by doxycycline to overexpress 4- to 5-fold either APE-1 wild type (wt), C65A (redox deficient), E96A (exonuclease deficient), or E96Q (exonuclease deficient) mutants and to down-regulate endogenous APE-1 by short hairpin RNA to 10% of the original level. Clonogenic results indicated that the induction of wt or C65A, but not E96A or E96Q, made cells approximately 2-fold resistant to L-OddC and β-L-2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine (L-Fd4C), whereas the down-regulation of APE-1 sensitized cells by approximately 2-fold to L-OddC and L-Fd4C. The alteration of APE-1 in cells did not change the sensitivity of these cells to β-D-2′,2′-difluorodeoxycytidine (dFdC; gemcitabine) and β-D-arabinofuranosylcytosine (AraC), both of which are D-configuration deoxycytidine analogs. The DNA incorporation of L-OddC, but not that of dFdC, was decreased by the induction of wt APE-1 but not E96A mutant and was increased by the down-regulation of APE-1. The rate of retention of L-OddC was inversely correlated to the level of APE-1 in isolated nuclei; however, this was not the case for dFdC. In conclusion, this study supports the hypothesis that APE-1 plays a critical role in the actions of L-configuration but not D-configuration nucleoside analogs.

β-L-Dioxolane-cytidine [L-OddC (BCH-4556, troxacitabine)] is a novel L-configuration deoxycytidine analog shown to have potential anticancer, anti-hepatitis B virus, and anti-human immunodeficiency virus activity by our laboratory (Grove et al., 1995; Grove and Cheng, 1996) and others (Kim et al., 1992). Clinical evaluation demonstrates its effectiveness against both leukemia and solid tumors. Phase II clinical studies have shown that L-OddC has significant anti-leukemic activity in patients with acute myeloid leukemia, chronic myelogenous leukemia in the blastic phase (Giles et al., 2002), and modest activity in advanced pancreatic adenocarcinoma (Lapointe et al., 2005).

Our previous studies showed that L-OddC can be phosphorylated by deoxycytidine kinase to its monophosphate metabolite which is further phosphorylated by cellular kinases to its di- and triphosphate metabolites (Grove and Cheng, 1996). The triphosphate form of L-OddC can be incorporated into DNA in vitro by DNA polymerases α, β, δ, γ, and ε (Kukhanova et al., 1995). Because L-OddC lacks a hydroxyl

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ABBREVIATIONS: L-OddC, β-L-dioxolane-cytidine (troxacitabine, BCH-4556); L-OddCMP, β-L-dioxolane-cytidine monophosphate; APE-1, apurinic/apyrimidinic endonuclease; L-Fd4C, β-L-2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine; dFdC, β-D-2′,2′-difluorodeoxycytidine (gemcitabine); AraC, β-D-arabinofuranosylcytosine; shRNA, short-hairpin RNA; DTT, dithiothreitol; PBS, phosphate-buffered saline; ANOVA, analysis of variance; wt, wild type; dFdCMP, β-D-2′,2′-difluorodeoxycytidine monophosphate; CRT0044876, 7-nitroindole-2-carboxylic acid; TCA, trichloroacetic acid.

group at the 3′-position, once incorporated, it causes premature termination of DNA replication, eventually leading to cell death. The amount of L-OddCMP at the DNA 3′ terminus correlated well with the cytotoxicity of L-OddC (Grove and Cheng, 1996). It was found that different tumor cells have different capacities to remove L-OddC from DNA once incorporated (Grove and Cheng, 1996).

We purified the most active enzyme capable of excising L-OddCMP from the DNA of nuclei from acute lymphocytic leukemia cells from patients in blast crisis (Chou et al., 2000). It was identified as apurinic/apyrimidinic endonuclease (APE-1), which is a key enzyme in the repair of apurinic/apyrimidinic sites in DNA. It also plays a role in regulating gene expression through its N-terminal redox domain by mediating the reductive activation of oxidized proteins (Evans et al., 2000). We showed that human apurinic/apyrimidinic endonuclease (APE-1) has an exonuclease activity capable of removing nucleosides from 3′ termini of DNA in vitro, preferentially removing L-OddC and other L-configuration nucleosides over D-configuration nucleosides (Chou and Cheng, 2000). The relative efficiency of removing various analogs from DNA is L-OddC (100%), L-Fd4C (78%), β-L-2′,3′-dideoxycytidine (60%), β-L-2′-deoxy-3′-thiacytidine (48%), 2′,3′-dideoxycytidine (12%), dFdC (9%), deoxycytidine (4%), and AraC (4%). We further discovered that APE-1 has a DNA 3′→5′-exonuclease activity not previously recognized on mismatched deoxyribonucleotides that is not only 25-fold more efficient in removing L-OddC but is also 10- to-20-fold more efficient in removing misincorporated nucleotides from the DNA 3′ terminus than the correctly incorporated nucleotide (Chou and Cheng, 2002). This suggests that APE-1 may play an important role in the proofreading of DNA polymerase β that is critical for AP site repair. Polymerase β has a relatively low fidelity of nucleotide incorporation and no intrinsic DNA exonuclease activity, but it could physically interact with APE-1. Our observation provided important information for furthering the characterization of 3′-5′ exonuclease activity of APE-1 (Shevelev and Hubscher, 2002; Wilson, 2003; Wong et al., 2003).

The importance of APE-1 in the cytotoxicity of L-OddC is supported by a study from Schild et al. (1999). They found that a Chinese hamster ovary cell line overexpressing human APE-1 displayed approximately 1.7-fold resistance to L-OddC compared with the Chinese hamster ovary parental cell line (Schild et al., 1999). This study did not address the issue of whether the L-OddC resistance was due to APE-1-associated exonuclease or other associated activity (i.e., the redox activity). We developed a set of tet-on RKO (human colorectal carcinoma) cell lines that could be induced by doxycycline to overexpress APE-1 wild type (wt), C65A (redox-deficient), E96A (exonuclease-deficient), or E96Q (exonuclease-deficient) mutants or induced by doxycycline to down-regulate endogenous APE-1 by short-hairpin RNA (shRNA). In these cell lines, we studied whether the level of APE-1 protein may modulate the cytotoxicity of L-configuration deoxycytidine analogs (L-OddC and L-Fd4C) versus D-configuration deoxycytidine analogs (dFdC and araC). We also examined the impact of the level of APE-1 protein on the incorporation and the retention of L-OddC in cellular DNA.

Materials and Methods

RKO Cell Line Establishment. RKO cells (human colorectal carcinoma) were grown in RPMI 1640 medium with 10% (tetracycline-free) fetal bovine serum (Clontech, Mountain View, CA) and were transfected with pcDNA6/TR vector. Permanent cell lines containing pcDNA6/TR were selected by 5 μg/ml blasticidin. In the APE-1 up-regulated cell lines, the fragment of DNA T7-APE-1 (N-terminal T7-tag) wt, C65A, E96A, or E96Q was amplified using PCR from the APE-1-expression vector (Chou and Cheng, 2003) and cloned into a pcDNA5/TO vector (Invitrogen, Carlsbad, CA). The APE-1-pcDNA5/TO vector was transfected into RKO-pcDNA6/TR cells. Hygromycin B, 150 μg/ml, was used to select permanent cell lines. In the down-regulated APE-1 cell lines, a shRNA sequence for down-regulation of APE-1 was designed by with the use of software provided by Invitrogen. The complementary DNA oligonucleotide APE-1 (5′-GCCTGGACTCTCTCATCAATAcgaaTATTGATGAGAGAGTCCAGGC-3′) (SENSE-loop-ANTISENSE) or control sequence (5′-ATGCATTCTAGTACCGGTAGGcgaaCCTACCGGTACTAGAATGCA-3′) was cloned into pENTR/H1/TO to express shRNA. Clones were selected using 500 μg/ml Zeocin (Invitrogen). Doxycycline was used to induce protein or shRNA expression. The level of protein expression and the homogeneity of protein expression were determined by Western blotting and confocal microscopy, respectively. Clones with homologous expression of APE-1 were isolated and expanded.

Western Blotting. Cells were lysed in 2× SDS sample buffer (62.5 mM Tris-HCl, pH 7.4, 2% SDS, 10% glycerol, 50 mM DTT, and 0.05% bromphenol blue) and sonicated for 10 s to shear DNA. The whole-cell extracts were then electrophoresed through 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) with a Miniprotein II transferring apparatus (Bio-Rad). The membranes were blocked and probed in PBS-Tween 20 buffer (1× PBS buffer, 0.2% Tween 20) containing 5% nonfat milk. APE-1 protein was detected using polyclonal APE-1 at 1:7500 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). Actin, the internal control used to confirm equal protein loading, was detected using a monoclonal actin antibody diluted 1:2500 (Sigma, St. Louis, MO). The membranes were then further incubated with horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (1:5000; Sigma), The immunoreactive bands were visualized by enhanced chemiluminescence reagents (PerkinElmer Life and Analytical Sciences, Boston, MA), and densitometry scanning was performed with the densitometer (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Confocal Microscopy. Doxycycline at 0.5 ng/ml or 100 ng/ml was used to induce APE-1 protein for 3 days or shRNA expression for 5 days. RKO cells (10^5) in 0.5 ml of RPMI 1640 medium containing 10% fetal bovine serum were seeded into a well of an eight-chamber slide and incubated overnight. Cells were fixed with 4% paraformaldehyde in PBS and then permeabilized with 0.5% Triton X-100 in PBS. To block nonspecific binding, 1% bovine serum albumin in PBS was used. Overexpressed T7-tagged APE-1 proteins and the endogenous APE-1 proteins were targeted by monoclonal T7-tag antibody (Novagen, Madison, WI) at 1:2000 dilution and monoclonal APE-1 antibody (Novus, Littleton, CO) at 1:3000 dilution, respectively, at room temperature for 1 h, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG at 1:200 dilution. Cytoplasmic actin was counterstained with 0.25 μg/ml of rhodamine phalloidin (Invitrogen). Cells were then sealed in antifade reagent (Invitrogen). Confocal micrographs were scanned by a laser scanning confocal microscope, LSM 510 (Carl Zeiss, Inc., Thornwood, NY).

AP Endonuclease Activity. RKO cell protein was extracted using a lysis buffer (50 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) and disrupted by sonication at 4°C. Cell debris was removed by centrifugation (13,000 rpm, 4°C, 20 min). Protein concentrations of the supernatants were quantitated by protein assay kit (Bio-Rad). Different

amounts of cell lysate were reacted with the 40-mer oligonucleotide containing AP site analog (tetrahydrofuran, f) 5′-labeled by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) using [γ^{32}P]ATP (PerkinElmer Life and Analytical Sciences) as the phosphate group donor. The labeling reaction was performed at 37°C for 20 min and unincorporated free ATP was removed with a G25 column (Roche Applied Science). The duplex oligonucleotide substrate for APE-1 was generated by mixing ^{32}P-labeled 40-mer and antisense 40-mer in 1× annealing buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA) and by boiling for 2 min followed by cooling slowly to room temperature. Oligonucleotide substrate (approximately 50 fmol) was incubated with APE-1 and various concentrations of cell lysate in a 10-μl reaction containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, and 0.1 mg/ml bovine serum albumin. The reaction was performed at 37°C for 3 min and terminated by adding 4 μl of loading buffer (98% formamide, 10 mM EDTA, 10 mM NaOH, 0.1% bromphenol blue, and 0.1% xylene cyanol). Samples were then boiled and analyzed by 20% denaturing urea/polyacrylamide gel electrophoresis. The amount of cleavage was quantified with a PhosphorImager (GE Healthcare) and the percentage of cleavage product was calculated from the fraction of cleavage bands versus total bands.

Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and the sequences are as follows: ^{32}Pgtggcgcggagacttagagafatttggcgcggggaattcc and caccgcgcctctgaatctctgtaaaccgcgccccttaagg.

Clonogenic Assay. Cells (1×10^3) that were pretreated with or without doxycycline at 100 ng/ml for 5 days were plated in six-well plates for 16 h. Cells were treated with L-OddC, L-Fd4C, dFdC, or AraC for 72 h followed by an exchange with fresh culture medium. The colonies were counted after incubating 7 days and then stained with methylene blue. The results are means and S.D. from three independent experiments.

Metabolism of Nucleoside Analogs. The cells were treated with [^{3}H]L-OddC at 500 nM (2 Ci/mmol) for 16 h and [^{3}H]dFdC at 500 nM (2 Ci/mmol) for 60 min. The cells were harvested in cold phosphate-buffered saline containing 20 μM dipyridamole (Sigma) and extracted with 15% trichloroacetic acid for 10 min on ice. The supernatant containing the nucleoside and its phosphorylated forms was extracted with a 45/55 ratio of trioctylamine and 1,1,2-trichlorotrifluroethane. The trichloroacetic acid insoluble pellet representing the nucleotide incorporated into the DNA was washed twice and resolubilized in Me_2SO before evaluation using a scintillation counter (LS5000TD; Beckman Coulter, Fullerton, CA). The nucleoside analog metabolites were analyzed by high-pressure liquid chromatography (Shimadzu, Braintree, MA) connected to a radiometric detector (Flow Scintillation Analyzer, 150TR; PerkinElmer Life and Analytical Sciences) using Partisil SAX column (Whatman, Clifton, NJ). All results are means and S.D. from at least three experiments.

Retention of Deoxycytidine Analogs in Nuclear DNA. The cells were treated with [^{3}H]L-OddC at 500 nM (2 Ci/mmol) for 16 h and [^{3}H]dFdC at 500 nM (2 Ci/mmol) for 60 min. The cells were harvested in ice-cold phosphate-buffered saline containing 20 μM dipyridamole (Sigma). Nuclei were isolated in an ice-cold nuclei isolation buffer [0.5% Nonidet P-40, 20 mM Tris-HCl pH 7.4, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, and 1× proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN)]. Nuclei were washed twice with ice-cold nuclei isolation buffer. The retention reaction was started by resuspending isolated nuclei into optimum APE-1 exonuclease reaction buffer (Chou and Cheng, 2003) and incubating in a 37°C water bath. The nuclei were pelleted at different time points using 15% TCA and the amount of L-OddCMP and dFdCMP present in DNA was determined by scintillation counting.

Animal Studies. Male NCR-nude mice (average body weight of 20 g), 4 weeks of age, were obtained from Taconic Farms Inc. and acclimated to laboratory conditions 1 week before tumor implantation. Nude mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. RKO tumor xenografts were established by injecting s.c. 2×10^6 RKO cells/mice (Con-shRNA, shRNA, Con-pcDNA5, wt-APE-1, C65A-APE-1, E96A-APE-1) with or without pretreatment with doxycycline 100 ng/ml for 6 days in culture. To keep APE-1 protein up- or down-regulated, 100 μg/ml of doxycycline was added to the drinking water that contains 1% sucrose for the mice. Measurement was initiated when the tumors were 100 to 250 mm^3, which is approximately 10 days after injection of RKO cells. RKO tumors were measured daily using a caliper, and the body weight of the mice was monitored for toxicity. Tumor volume was estimated by using the formula length × width^2 × π/6.

Statistical Analysis. Data were analyzed by two-way ANOVA (Prism 4; GraphPad Software, San Diego, CA), Student's t test (Microsoft Excel), and one-way ANOVA (GraphPad Prism). The difference was considered to be statistically significant when $P < 0.05$.

Results

Up-Regulation and Down-Regulation APE-1 Protein Expression and Its Mutants. We established tet-on RKO (human colorectal carcinoma) cell lines that could be induced by doxycycline to overexpress APE-1 wild type (wt), C65A (redox-deficient), E96A (exonuclease-deficient), or E96Q (exonuclease-deficient) mutants or induced by doxycycline to down-regulate endogenous APE-1 by shRNA. Western blotting indicated that T7 tagged-APE-1 proteins (wt, C65A, E96A, and E96Q) could be up-regulated or endogenous APE-1 could be down-regulated by doxycycline in a dose-dependent manner (Fig. 1A). The maximum expression of T7-tagged-APE-1 proteins (4- to 5-fold compared with endogenous APE-1) could be found by adding doxycycline at 100 ng/ml for 72 h (data not shown), whereas the maximum knockdown of the endogenous APE-1 (approximately 10% remaining compared with the control) could be found by adding doxycycline at 100 ng/ml for 6 days (Fig. 1A). Reports indicated that APE-1 could form a complex with heterogeneous nuclear ribonucleoprotein L, bind to one of its own regulatory elements (nCaREB2), and repress its own expression, thus providing a means to control its intracellular level (Kuninger et al., 2002). Our results showed that the expression of T7-tagged-APE-1 proteins, wt type and its mutants, did not seem to affect the endogenous level of APE-1. Perhaps APE-1 is not the rate-limiting factor for the negative feedback regulation for its expression in RKO cells.

Subcellular Localization of APE-1 Protein Cells with Different APE-1 Protein Expression. In terms of subcellular localization, APE-1 is found primarily in the nucleus, however, in different tissues or stimulating conditions, it can also localized to other cellular compartments, including the cytoplasm and mitochondria. (Tell et al., 2005). Our confocal pictures indicated that in both overexpression and underexpression conditions, APE-1 accumulated primarily in the nucleus and responded to doxycycline in a dose-dependent manner (Fig. 1B). The T7-tag did not affect the subcellular localization of APE-1. The C65A (data not shown), E96A, and E96Q (data not shown) mutations did not affect the subcellular localization of APE-1 protein. This enabled us to study the relationship between the level of APE-1 protein in the nucleus and the cytotoxicity of nucleoside analogs.

The Impact of APE-1 Protein Levels on Cell Growth. Overexpression of APE-1 wt, C65A, E96A, and E96Q mu-

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tants did not have a significant impact on RKO cell growth in culture (result not shown). RKO cell growth was not affected by down-regulating APE-1 90% with shRNA for up to 1 month in culture (result not shown). However, in a xenograft model, the down-regulation of APE-1 protein could reduce the rate of tumor growth by approximately 50% (Supplemental Fig. 1). Overexpression of wt APE-1, C65A, or E96A did not significantly affect the tumor growth in the xenograft model (Supplemental Fig. 1).

The Impact of APE-1 Protein Level on Cellular Endonuclease Activity. We studied the relationship of the APE-1 protein level and the cellular endonuclease activity. Our results indicated that manipulation of the APE-1 protein levels did have an impact on the cellular endonuclease activity (Fig. 2). Up-regulation of wt and C65A, which have similar endonuclease and exonuclease activity (Chou and Cheng, 2003), but not E96A or E96Q, which are endonuclease- and exonuclease-deficient mutants, increased the endonuclease activity by 4-fold in the total cell lysate. Down-regulation of APE-1 protein to 10% of its original level by shRNA decreased the endonuclease activity in cell lysates by 6-fold.

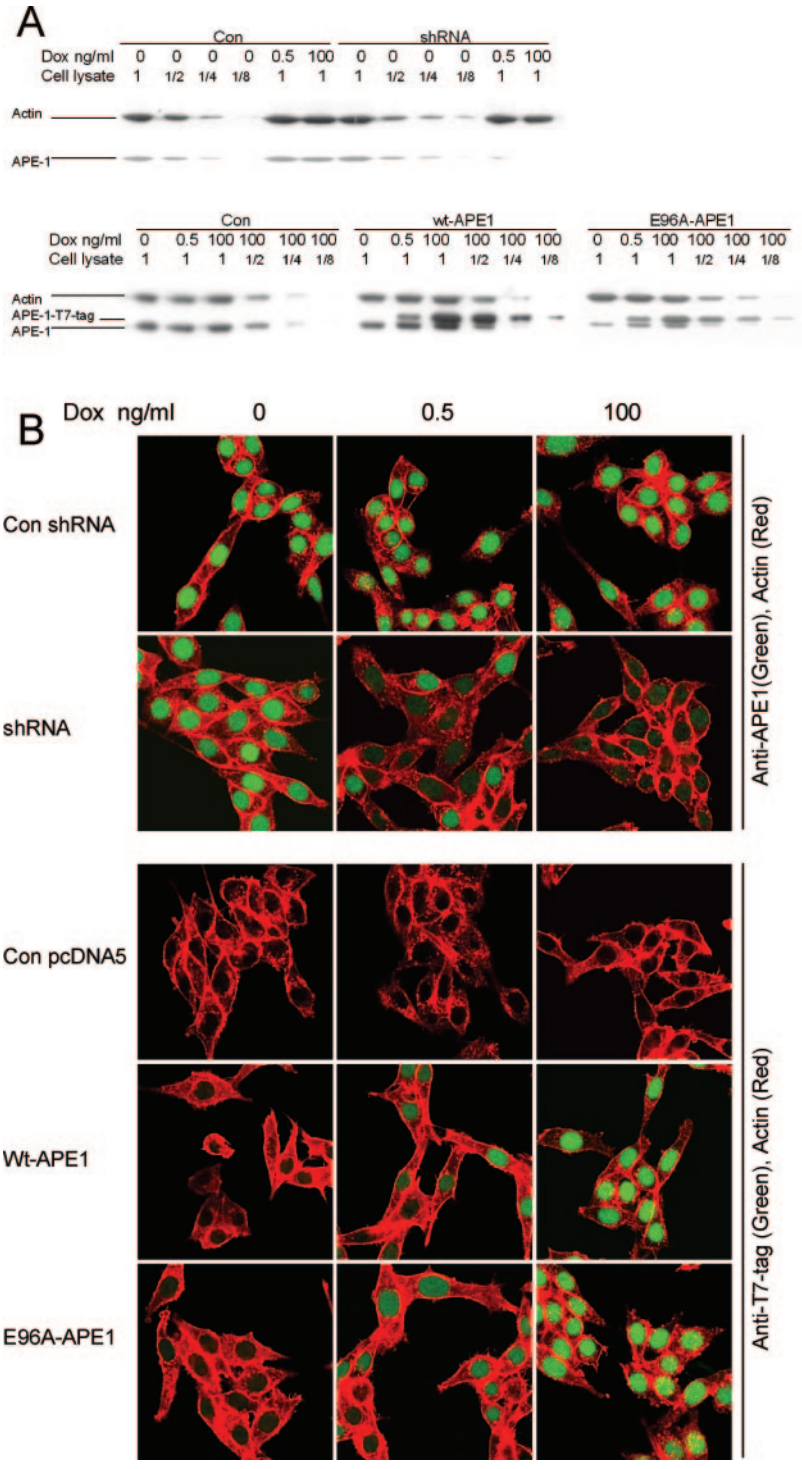
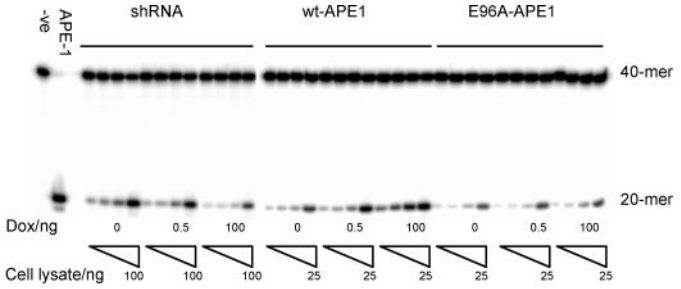


Fig. 1. Characterization of RKO cell lines stably transfected with inducible shRNA of APE-1, T7-tagged wt-APE1, or E96A-APE-1 mutant. A, the quantitative analysis of APE-1 protein in different cell lines pretreated with different concentrations of doxycycline by Western blotting. B, confocal microscopy showing the subcellular localization and expression of APE-1 from different cell lines at different concentrations of doxycycline.

The Impact of APE-1 Protein Expression Level on the Cytotoxicity of L-Configuration Nucleoside Analogs (L-OddC and L-Fd4C) and D-Configuration Nucleoside Analogs (dFdC and AraC) toward RKO Cells. The impact of APE-1 protein on the cytotoxicity of L-configuration nucleoside analogs (L-OddC, L-Fd4C) and D-configuration deoxycytidine analogs (dFdC, AraC) was compared using clonogenic assays. Results indicated that 4-fold up-regulation of wt or C65A made RKO cells 2-fold resistant to L-OddC and L-Fd4C ($P < 0.05$, t test) but not to dFdC and AraC (Table 1). However, this was not the case with E96A or E96Q mutants, which have 1000- and 300-fold lower exonuclease activity compared with that of wt, respectively. In contrast, down-regulation of APE-1 protein to approximately 10% by shRNA sensitized cells (2-fold) to L-OddC and L-Fd4C ($P < 0.05$, t test) but again not dFdC and AraC (Table. 1). Western blot results indicated that the levels of overexpression of wt and mutated APE-1 and the levels of underexpression of APE-1 were constant during the treatment of these compounds (Supplemental Fig. 2). The clonogenic assay results support our previous findings that APE-1 preferentially removed L-configuration over D-configuration nucleoside analogs. This suggests that the exonuclease activity of APE-1 may be an important factor in the drug sensitivity of L-configuration nucleosides.

The Impact of APE-1 Protein Expression Level on the Incorporation of L-OddC and dFdC into Cellular DNA. We showed that the cytotoxicity of L-OddC is directly related to the steady-state level of the incorporated L-OddC in nuclear DNA (Grove and Cheng, 1996). To determine whether the exonuclease activity of APE-1 plays a direct role on the cytotoxicity of L-configuration nucleoside analogs, the incorporation of nucleoside analogs into nuclear DNA was examined. Our results indicated that the overexpression of APE-1 wt, but not E96A, decreased the incorporation of L-OddC into nuclear DNA ($P < 0.05$, one-way ANOVA), whereas the underexpression of APE-1 increased the amount of L-OddC incorporation into nuclear DNA ($P < 0.05$, one-way ANOVA) (Fig. 3A). The expression levels of APE-1 did not affect the incorporation of dFdC into DNA (Fig. 3B). There was no change in L-OddC and dFdC metabolism in cells that had down-regulation of endogenous APE-1 protein or overexpression of wt and E96A (Fig. 3, C and D). The level of PGK protein which phosphorylates L-OddCDP to L-OddCTP was also not changed when the level of APE-1 protein was altered (results not shown).

The Impact of APE-1 Protein Expression Level on the Retention of L-OddC and dFdC in Isolated Nuclei from L-OddC- and dFdC-Pretreated Cells. To study whether the changes in incorporation of L-OddC into nuclear DNA at different cellular APE-1 levels is due to the changes of APE-1 exonuclease activity, we examined the retention of L-OddC and dFdC in nuclei versus time. Nuclei from L-OddC and dFdC pretreated cells, which had different APE-1 levels, were isolated in a 0.5% Nonidet P-40 buffer. Nuclei were incubated in the optimum APE-1 exonuclease buffer for the desired time. Results indicated that the down-regulation of APE-1 increased the retention of L-OddC in nuclear DNA at different time points ($P < 0.05$, two-way ANOVA) (Fig. 4A). In contrast, up-regulation of wt APE-1 but not E96A APE-1, which has 1000-fold lower exonuclease activity, decreased the retention of L-OddC in DNA versus time ($P < 0.05$, two-way ANOVA) (Fig. 4, B and C). Unlike L-OddC, the removal rate of dFdC from isolated nuclei was very slow and independent of APE-1 levels (Fig. 4, D–F).



	Relative Cellular AP Endonuclease Activity		
	Dox ng/ml		
Cell line with transfection	0	0.5	100
Con-shRNA	1	1	1
shRNA	1	0.5	0.16
Con pCNDA5	1	1	1
Wt-APE1	1	2	4
C65A-APE1	1	1.5	3.5
E96A-APE1	1	1	1

Fig. 2. The doxycycline-dependent AP endonuclease activity of different APE-1 regulated cell lines. Top, 2-fold-serial dilutions of cell lysates from cells pretreated with different concentrations of doxycycline reacted with 40-mer oligonucleotide containing an AP site at 37°C for 3 min. Purified recombinant APE-1 protein was added as positive control. Bottom, relative cellular AP endonuclease activity of different cell lines with different concentrations of doxycycline treatment. The rest of the procedures were the same as described under *Materials and Methods*.

Discussion

The cytotoxicity of L-configuration and D-configuration nucleoside analogs is controlled by several critical steps: the uptake of the nucleoside into cells, phosphorylation of the nucleoside stepwise to its triphosphate metabolites, incorporation of the nucleoside triphosphate into the 3′ terminus of DNA, and the removal of the nucleoside from DNA. However, the key enzymes involved in the different steps of the metabolism of L- and D-configuration nucleoside analogs are quite different. L-OddC has been reported to be taken up into cells primarily by passive diffusion and to a lesser extent by nucleoside transport carriers (Gourdeau et al., 2001, 2004), whereas dFdC is taken up primarily by nucleoside carriers (Achiwa et al., 2004). Once inside the cell, both nucleoside analogs are phosphorylated by deoxycytidine kinase to their monophosphate metabolites, although the efficiencies of these reactions are quite different (Grove and Cheng, 1996). L-OddCMP and dFdCMP could be phosphorylated in vitro by recombinant CMP/UMP kinase to L-OddC diphosphate and dFdC diphosphate, respectively. In vitro and in cells, all diphosphate metabolites of L-nucleoside analogs L-OddC, lamivudine, and clevudine, studied so far by us, were found to be phosphorylated by phosphoglycerate kinase rather than

nucleoside diphosphate kinase, nm23-H1, or nm23-H2 (Krishnan et al., 2002a,b, 2003). Several other antiviral nucleoside analogs, diphosphate metabolites, were also found to be phosphorylated by this enzyme in vitro (Gallois-Montbrun et al., 2004), whereas dFdC was found to be phosphorylated by nucleoside diphosphate kinase, nm23-H1, and nm23-H2 (Richardson et al., 2004).

The key enzymes for removal of L- and D-nucleoside ana-

TABLE 1

Inhibition of clonogenicity (LC_{50}) by L-configuration nucleoside analogs and D-configuration nucleoside analogs in RKO cell lines with under- or over-expression of APE-1 protein

LC_{50} was defined as the concentration of drug to give 50% of surviving fraction, and values are means ± S.D. of three experiments, with each data point done in triplicate.

Cell Line with Transfection	Dox (100 ng/ml)	Inhibition of Clonogenicity LC_{50}			
		L-Configuration		D-Configuration	
		L-OddC	L-Fd4C	dFdC	AraC
		nM	*μM*	*nM*	*nM*
Con shRNA	−	158 ± 18	5.4 ± 1.8	2.4 ± 0.5	16.5 ± 3.5
	+	163 ± 19	5.9 ± 2.3	2.5 ± 0.7	15 ± 4.5
shRNA	−	119 ± 20	6.1 ± 1.1	3.2 ± 1.1	20.7 ± 4.7
	+	56 ± 5	3.2 ± 0.9	3.4 ± 1.2	21.7 ± 5.6
Con pcDNA5	−	125 ± 35	5.5 ± 0.7	2.5 ± 0.6	12.2 ± 3.8
	+	128 ± 25	6.5 ± 0.6	2.6 ± 0.8	13 ± 2.8
Wt-APE-1	−	108 ± 25	4.3 ± 1.2	2.5 ± 0.4	16.5 ± 6.5
	+	238 ± 53	9.2 ± 2.7	2.6 ± 0.3	17.5 ± 2.5
C65A-APE-1	−	158 ± 25	5.6 ± 1.4	2.5 ± 0.1	18.5 ± 3.5
	+	263 ± 18	10.9 ± 2.3	2.7 ± 0.2	21 ± 1.4
E96A-APE-1	−	123 ± 4	4.5 ± 0.4	2.5 ± 0.1	14 ± 5
	+	130 ± 7	4.3 ± 0.5	2.6 ± 0.1	15 ± 6
E96Q-APE-1	−	140 ± 27	4.9 ± 0.5	2.4 ± 0.3	19 ± 3
	+	133 ± 19	5.2 ± 0.3	2.2 ± 0.2	17 ± 5

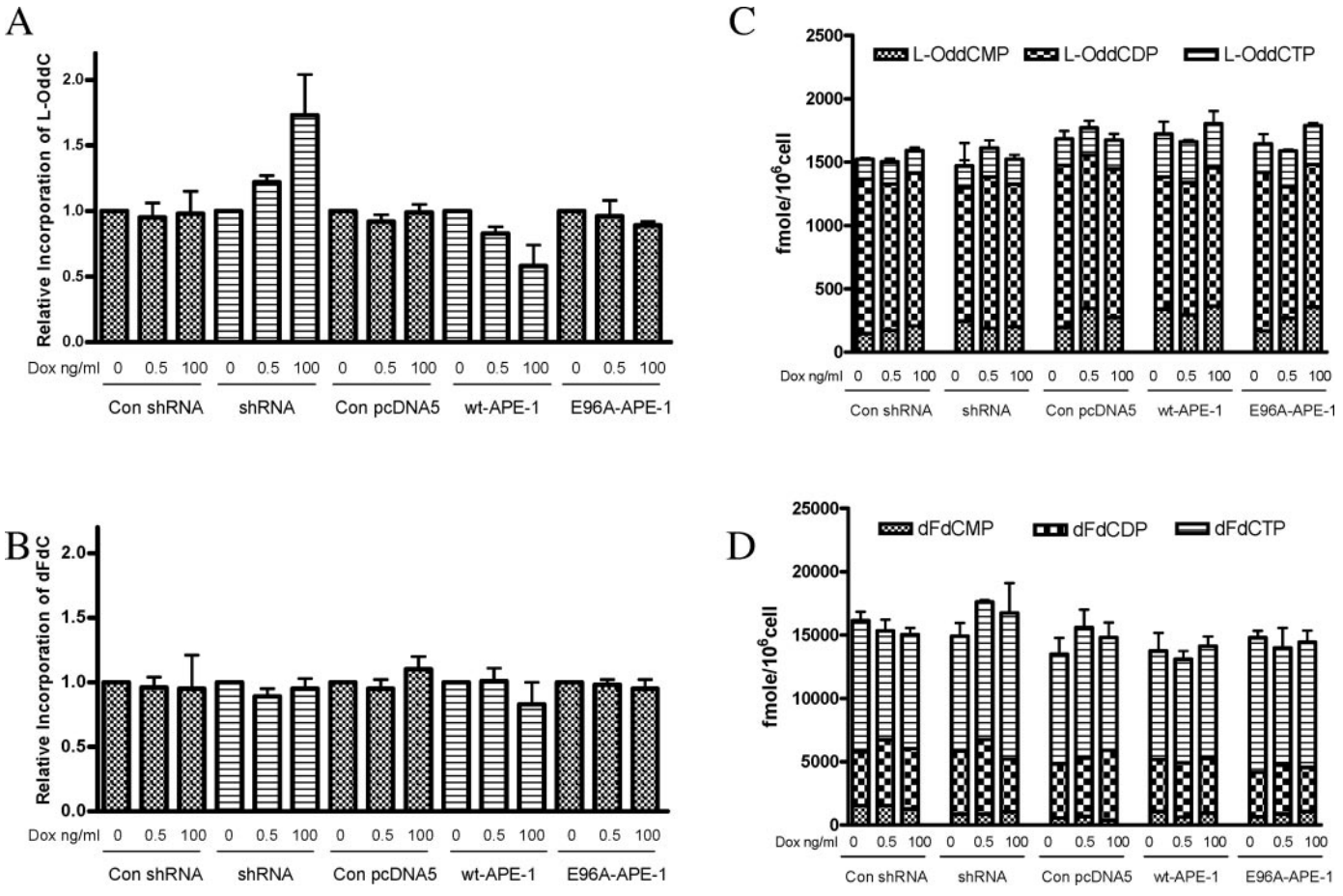


Fig. 3. A and B, the doxycycline-dependent incorporation of L-OddC and dFdC into cellular DNA of different APE-1 regulated cell lines. C and D, the doxycycline-dependent metabolism of L-OddC and dFdC in different APE-1 regulated cell lines. Cells treated with different concentrations of doxycycline were incubated with [^{3}H]L-OddC at 500 nM (2 Ci/mmol) for 16 h and [^{3}H]dFdC at 500 nM (2 Ci/mmol) for 60 min. TCA (15%) was used to precipitate macromolecules in the cells, the amount of L-OddCMP and dFdCMP present in DNA was determined by scintillation counting. The metabolites of L-OddC and dFdC in TCA soluble fraction were separated by high-performance liquid chromatography. The rest of the procedures were the same as described under *Materials and Methods*.

logs from 3′ terminus of DNA are also different. In vitro studies have shown that the exonuclease activity of Trex1 could remove AraCMP and dNMP in vitro (Mazur and Perrino, 1999), but the exonuclease activity of p53 has preferences for the removal of D-configuration nucleoside analogs over L-configuration analogs in vitro (Kukhanova et al., 2000; Pelicano et al., 2000). We reported that APE-1 has preferences in the removal of L-nucleoside analogs over D-nucleoside analogs from DNA at the 3′ end in vitro (Chou and Cheng, 2000). By using a set of tet-on cell lines in which APE-1 protein can be either up-regulated or down-regulated by the addition of doxycycline, we found that the level of APE-1 protein inside the cells was inversely correlated to the cytotoxicity of L-OddC and L-Fd4C but not that of dFdC and AraC. These results support our previous report that APE-1 has a much higher relative efficiency of removing L-OddC (100%) and L-Fd4C (78%) than dFdC (9%) and AraC (4%) (Chou and Cheng, 2000). Furthermore, the level of APE-1 protein also has a big impact on the incorporation and retention of L-OddC into DNA but not dFdC. These results provide strong evidence that APE-1 exonuclease activity plays an important role in the removal of L-configuration nucleoside analogs from DNA inside the cell and can be a determining factor in the drug resistance of L-configuration nucleoside analogs.

Previous reports demonstrated the importance of APE-1 during early embryonic development and growth of mice (Xanthoudakis et al., 1996; Ludwig et al., 1998). Recent reports indicated that down-regulation of APE-1 in some cancer lines by small interfering RNA can slow the growth of some cancer cells in culture (Fung and Demple, 2005). These reports support the idea that APE-1 is important for DNA repair and cell growth. In our tet-on RKO cell system, the down-regulation of APE-1 did not significantly affect the growth of RKO cells (several clones) in culture, but the down-regulation of APE1 significantly slows tumor growth in vivo (xenograft model) (Supplemental Fig. 1). This implies that APE-1 may play a key role in controlling certain factors required for tumor growth of RKO cells in vivo. Indeed, beyond DNA damage repair, APE-1 has been identified as a protein capable of nuclear redox activity, inducing the DNA-binding activity of many transcriptional factors, such as activator protein 1, nuclear factor-κB, Myb, polyoma virus enhancer binding protein 2, nuclear factor Y, epidermal growth factor receptor 1, hypoxia-inducible factor α, activating transcription factor/cAMP response element-binding protein family, p53, and Pax proteins (Evans et al., 2000; Tell et al., 2005). Some of these factors could be important for RKO cell growth in vivo. Because APE-1 activity could also be affected by post-translational modifications such as phosphorylation and acetylation (Evans et al., 2000; Tell et al., 2005), these modifications could also have an influence on the minimum level of APE-1 required for cells to survive. It would be worthwhile to compare the post-translational modification of APE-1 in vitro and in vivo.

Cell culture studies have already shown that nuclei AP activity can be affected by several factors: the protein expression level, amino acid polymorphism, subcellular distribution, post-translational modification, phosphorylation, and acetylation (Evans et al., 2000; Tell et al., 2005). In clinical studies, it also has been shown that different types of tumors have different levels of APE-1 protein, different amino acid polymorphisms and different patterns of subcellular distribution (Hu et al., 2001; Silber et al., 2002; Bobola et al., 2004, 2005; Ito et al., 2004, Wang et al., 2004). The impacts of these factors on the AP endonuclease activity of APE-1 have been

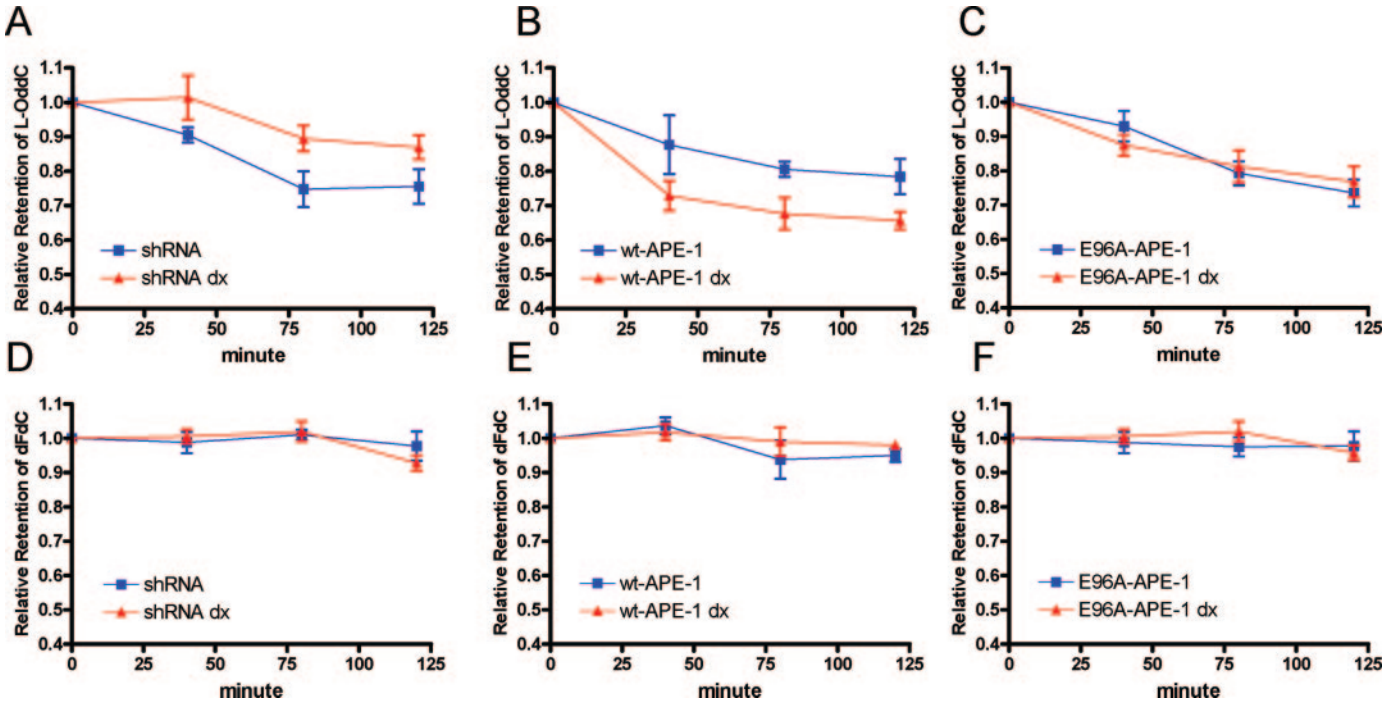


Fig. 4. The doxycycline-dependent retention of L-OddC (A–C) and dFdC (D–F) in cellular DNA of different APE-1-regulated cell lines. Cells treated with or without doxycycline 100 ng/ml were incubated with [^{3}H]L-OddC at 500 nM (2 Ci/mmol) for 16 h and [^{3}H]dFdC at 500 nM (2 Ci/mmol) for 60 min. Nuclei were isolated by 0.5% Nonidet P-40 buffer and then incubated in optimum APE-1 exonuclease reaction buffer (Chou and Cheng, 2003). TCA (15%) was used to precipitate the nuclei at different time points, the amount of L-OddCMP and dFdCMP present in DNA was determined by scintillation counting. The rest of the procedures were the same as described under *Materials and Methods*.

found to affect the clinical outcome of chemotherapy and radiotherapy. It is likely that these factors may also modulate the exonuclease activity of APE-1 and thus will affect the clinical outcome of L-configuration nucleoside therapy. Further investigations regarding these factors on the exonuclease activity of APE-1 should be made.

The results from this study and our previous reports indicate that inhibition of APE-1 exonuclease activity, which can excise L-OddC from DNA, could increase L-OddC cytotoxicity. Our previous report has indicated that Gp4G, a natural dinucleotide that exists in mammalian cells, can inhibit APE-1 exonuclease activity (Chou and Cheng, 2003). However, the regulation of intracellular of Gp4G is not clear. A chemical inhibitor, CRT0044876, has been identified to have inhibitory effects on the AP endonuclease, 3′-phosphodiesterase, and 3′-phosphatase activities of APE-1 and is a specific inhibitor of the exonuclease III family of enzymes to which APE-1 belongs (Madhusudan et al., 2005). CRT0044876 could potentiate the cytotoxicity of several DNA base-target compounds. It would be interesting to explore whether CRT0044876 could also inhibit APE-1 exonuclease activity and potentiate the cytotoxicity of L-configuration nucleoside analogs.

In conclusion, this report provides strong evidence to support the fact that APE-1 exonuclease activity plays an important role in the cytotoxicity of L-OddC and perhaps other L-deoxynucleoside analogs.

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