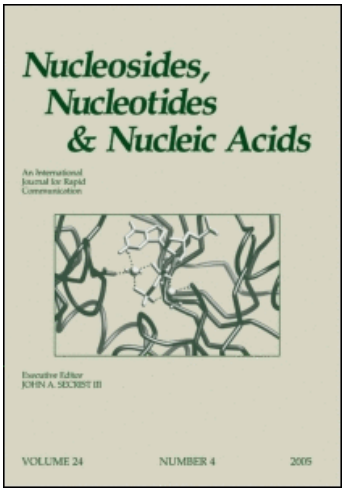

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Marina Kukhanova[a]; Tsung-Wu Liu[a]; Helene Pelicano[a]; Yung-Chi Cheng[a]
[a] Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

To cite this Article Kukhanova, Marina , Liu, Tsung-Wu , Pelicano, Helene and Cheng, Yung-Chi(2000) 'Excision of β-L- and β-D-Nucleotide Analogs From DNA by p53 Protein', Nucleosides, Nucleotides and Nucleic Acids, 19: 1, 435 — 446
To link to this Article: DOI: 10.1080/15257770008033019
URL: http://dx.doi.org/10.1080/15257770008033019

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(1&2), 435–446 (2000)

EXCISION OF β-L- and β-D-NUCLEOTIDE ANALOGS FROM DNA by p53 PROTEIN

Marina Kukhanova, Tsung-Wu Liu, Helene Pelicano, Yung-Chi Cheng*
Department of Pharmacology, Yale University School of Medicine, 333 Cedar St, New Haven, Connecticut 06520

Dedicated to the memory of Dr. Gertrude B. Elion

ABSTRACT. The tumor suppressor p53 protein plays a critical role in the cell-cycle progression. The role of the 3'-to-5' exonuclease activity of p53 protein in the DNA repair process remains elusive. Using an *in vitro* exonuclease assay and defined oligonucleotides terminated with β-D- and β-L-nucleoside analogs at the 3'-terminus, we studied the ability of p53 protein to excise β-L- and β-D-nucleoside analogs which have anticancer or antiviral potential. p53 protein removes β-D-nucleoside analogs more efficiently compared to that of β-L-nucleoside analogs. The affinity of p53 protein for an β-L-nucleotide terminated primer was 5 fold lower compared to non-modified primer. The hypothesis on an important role of the 3'-to-5' exonuclease activity of p53 protein in the action of nucleoside analogs was proposed.

The p53 protein is recognized as an important regulator of cell-cycle progression.[1,2] Intracellular level of p53 protein increases dramatically in response to a variety of DNA damaging agents following the arrest of cell-cycle in G1 phase.[2-4] Recent studies have shown that the incorporation of nucleoside analogs, such as gemcitabine or bromodeoxyuridine, into DNA increases the p53 protein level in cell nuclei as well.[5] The role of p53 protein in response to DNA damaging agents or inhibitors of DNA synthesis was thought to be partly due to its ability to recognize DNA damage and activate genes responsible for the DNA repair. The p53 protein mutations or deletions contribute to the formation of a wide range of human tumors.[6] Recently p53 protein was reported to exert a 3'-to-5' exonuclease activity,[7] which might play an important role in the removal of nucleoside analogs, used as anticancer drugs from the 3'-end of DNA. The exonuclease activity of p53 protein might also provide the higher selectivity of

action of anticancer nucleoside analogs against cancer cells, which have deletion or mutation in p53 protein, compared to normal cells. Furthermore, the induction of p53 protein in cells could also decrease the toxicity of anticancer or antiviral nucleoside analogs if these analogs are incorporated into the 3'-termini of cellular DNA. The structure-activity relationship between nucleoside analogs incorporated into DNA and the 3'-to-5' exonuclease of p53 protein is not clear. Among nucleoside analogs, 1-β-D-arabinofuranosylcytosine (araC), 1-β-D-arabinofuranosyladenosine (araA), and several β-L-nucleosides were found to be useful in both cancer and viral treatment. In this manuscript, we describe the substrate specificity of the 3'-to-5' exonuclease of p53 protein with a special focus on some β-L- and β-D-nucleoside analogs which have anticancer or antiviral potential.

EXPERIMENTAL PROCEDURE

Materials. dNTPs were obtained from Pharmacia LKB Biotechnology Inc. The D- and L-enantiomers of 2', 3'-dideoxy-3'-thiocytidine (SddC) as well as 3'-dideoxy-3'-dioxolane-cytidine (β-L-OddC) were provided by Dr. C.K.Chu, Department of Medicinal Chemistry, University of Georgia. Triphosphate forms of these compounds were prepared in this laboratory as described previously.[8] 2',3'-dideoxy-2', 3'-didehydro-5-fluorocytidine 5'-triphosphate (β-L-Fd4CTP) was synthesized at Vion Pharmaceuticals, Inc., New Haven, CT; araATP and araCTP were obtained from Sigma. Monoclonal antibodies PAb421 and Pab240 were purchased from Oncogene Science, Inc. A recombinant baculovirus that expressed the wild-type form of human p53 protein and Sf9 insect cells were provided by Dr. Wen-Hwa Lee, Center for Molecular Medicine, University of Texas. Hi-Trap Heparin-Sepharose columns were obtained from Pharmacia; cellulose phosphate (P11) was obtained from Whatman; 15-mer oligodeoxynucleotide d(ATTTCCTTAACTGCC)-5' was synthesized on an Applied Biosynthesis 380A DNA synthesizer at the Yale oligonucleotide Synthesis Facility and purified by a 20% PAGE containing 7M urea. Oligonucleotide was 5'-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase according to a standard procedure.[9]

Purification of p53 protein and measurement of the 3'-to-5' exonuclease activity. The crude cellular lysate from Sf9 cells infected with p53 containing baculovirus was

prepared according to a published procedure.[7] The lysate containing 40 mg protein was diluted ten-fold with buffer A (30 mM $KHPO_4$, pH 8.5, 1 mM EDTA, 2 mM dithiotreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and benzamidine each, 1 μg/ml leupeptin and pepstatin each, 0.2% aprotinin, 0.1% E64), and applied to a 5 ml Hi-Trap Heparin-Sepharose column. The bound protein was eluted with a 12 column volume linear gradient 0.05M - 1M KCl in buffer A and assayed for the 3'-to-5' exonuclease activity. The p53 protein was identified by Western blot analysis using antibodies Pab240. Those fractions that revealed both the 3'-to-5' exonuclease activity and a positive signal in Western blot analysis were combined, diluted 10 fold with buffer A, and applied to a 2 ml P11 column. Bound protein was eluted with a 20 column volume linear gradient 0.05M - 0.8M KCl in buffer A. Fractions of p53 protein were pooled, dialyzed against buffer A containing 50 mM KCl and applied on a Mono S HR 5/5 column. The p53 protein was eluted with a linear gradient 0.05M - 0.6M KCl in buffer A. The purity of p53 protein was assessed by SDS PAGE followed by silver-staining and mass-spectrum analysis. The 3'-to-5' exonuclease activity was assayed in the reaction mixture containing 50 mM Tris-acetate buffer, pH 8.5, 10 mM $MgCl_2$, 1 mM dithiotreitol, and 0.5 nM 5'-[^{32}P]-15-mer oligonucleotide in a total volume of 10 μl. Reactions were initiated by addition of 2-5 ng of p53 protein and allowed to proceed for selected times at 37°C. The reaction products were separated by a 15% denaturing PAGE as described elsewhere.[10]

Preparation of oligodeoxynucleotides with ddNMP analogs at the 3'-termini. The radiolabeled 15-mer oligonucleotide terminated at its 3'-end with the appropriate nucleoside analog was obtained using terminal nucleotidyltransferase (Gibco BRL). The reaction mixtures contained 10 pmol of 5'[^{32}P]-15-mer, 20 μM dNTP analog, 10 units of enzyme, and buffer provided by the manufacturer in a total volume of 20 μl. After incubation for 30-60 min at 37°C, the enzyme was heat-inactivated for 10 min at 70°C; the reaction mixtures were passed through a 1 ml Spin column with Sephadex G-25 (Pharmacia), and the products were used as substrates for study of the 3'-to-5'exonuclease.

Preparation of oligonucleotides with arabinofuranosyl nucleotides at the 3'-terminus. Oligonucleotides with araCMP or araAMP residues at the 3'-terminal were

prepared as described above with some modification. Due to incorporation of 3-4 arabinofuranosyl residues into the 3'-end of oligonucleotides by terminal nucleotidyltransferase, the reaction products were separated by a 20% denaturing PAGE. Bands corresponding to 16-mer and 17-mer oligonucleotides, which indicate the incorporation of one or two arabinosyl residues, were cut from gel, extracted with buffer (10 mM Tris-HCl buffer, pH 7.5, 0.5 mM EDTA), and purified using Sap-Pak columns (Waters Co.) according to manufacturer procedure.

Competitive binding analysis of p53 protein to β-L-OddCMP terminated primer. The reaction mixture contained 2 nM 5'-[^{32}P]-15 mer primer, p53 protein, buffer, as indicated above, and β-L-OddCMP terminated 15-mer primer at concentration ranges as shown in the Legend to FIG. 4. The reactions were proceeded for 15 min at 37°C.

RESULTS

Characterization of the purified p53 protein. The p53 protein was purified from Sf9 insect cells infected with a human p53-recombinant baculovirus using Hi-Trap Heparin Sepharose, P11, and Mono S HR 5/5 column chromatographic steps as described in Materials and Methods. In all steps p53 protein was identified by Western blot analysis using antibodies Pab240, and the exonuclease activity was assayed by PAGE analysis of the degradation products of 5'-^{32}P-oligonucleotide. Only those fractions that showed both p53 protein and the 3'-to-5'exonuclease activity were combined and applied on the next column. After the last column p53 protein appeared as a homogeneous band on a silver-stained gel with no contaminating protein bands (FIG.1b). The purity of the protein was also verified by mass-spectrum analysis (FIG.1a). Mass-spectrum analysis showed two peaks. The major peak with molecular weight 43924 Da presents the phosphorylated form of p53 protein with an average molar ratio of phosphate residues to protein of 3.5 : 1. The minor peak is p53 protein partly degraded during sample preparation. No other proteins were found by this method. Both exonuclease activity and p53 protein were depleted by 90% after the immunoprecipitation with antibodies Pab421 (data not shown). This highly purified p53 protein showed the 3'-to-5' exonuclease activity. As was demonstrated earlier,[7] the exonuclease activity of p53 protein was inhibited by 5 mM GMP. We have shown that the exonuclease activity was inhibited by inosine 5'-monophosphate as well with ID_{50} about 2 mM. The 3-to-5'

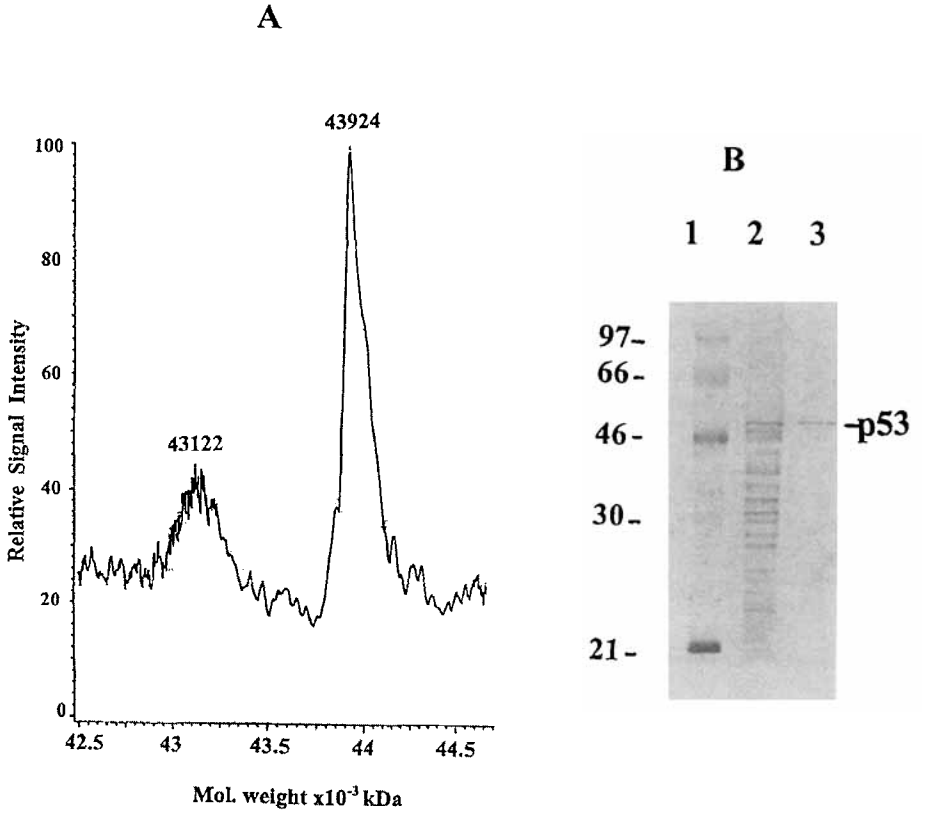

FIG. 1. (A) Mass-spectrum analysis of purified p53 protein. (B) SDS-PAGE analysis of p53 protein. Samples were run on a 0.75-mm thick gel, which was then silver-stained. Lane 1 - marker proteins with molecular weight shown on the left. Lane 2 - crude extract of Sf9 cells expressing p53 protein. Lane 3 - silver staining of p53 protein after the final step of purification.

exonuclease is rather stable and did not loose activity after incubation of p53 protein at 37°C up to 2 hr (data not shown).

Substrate specificity of the 3'-to-5' exonuclease activity of the p53 protein. The ability of the 3'-to-5' exonuclease activity of p53 protein to remove chain terminators from the 3'-end of DNA was examined on a 5'-[^{32}P]-15-mer primer with β-D-ddNMP, β-D-SddCMP, β-L-OddCMP, β-L-SddCMP, β-L-Fd4CMP, araAMP, or araCMP at the 3'-end. FIG. 2 shows the time-dependence of degradation of the 15-mer oligonucleotide

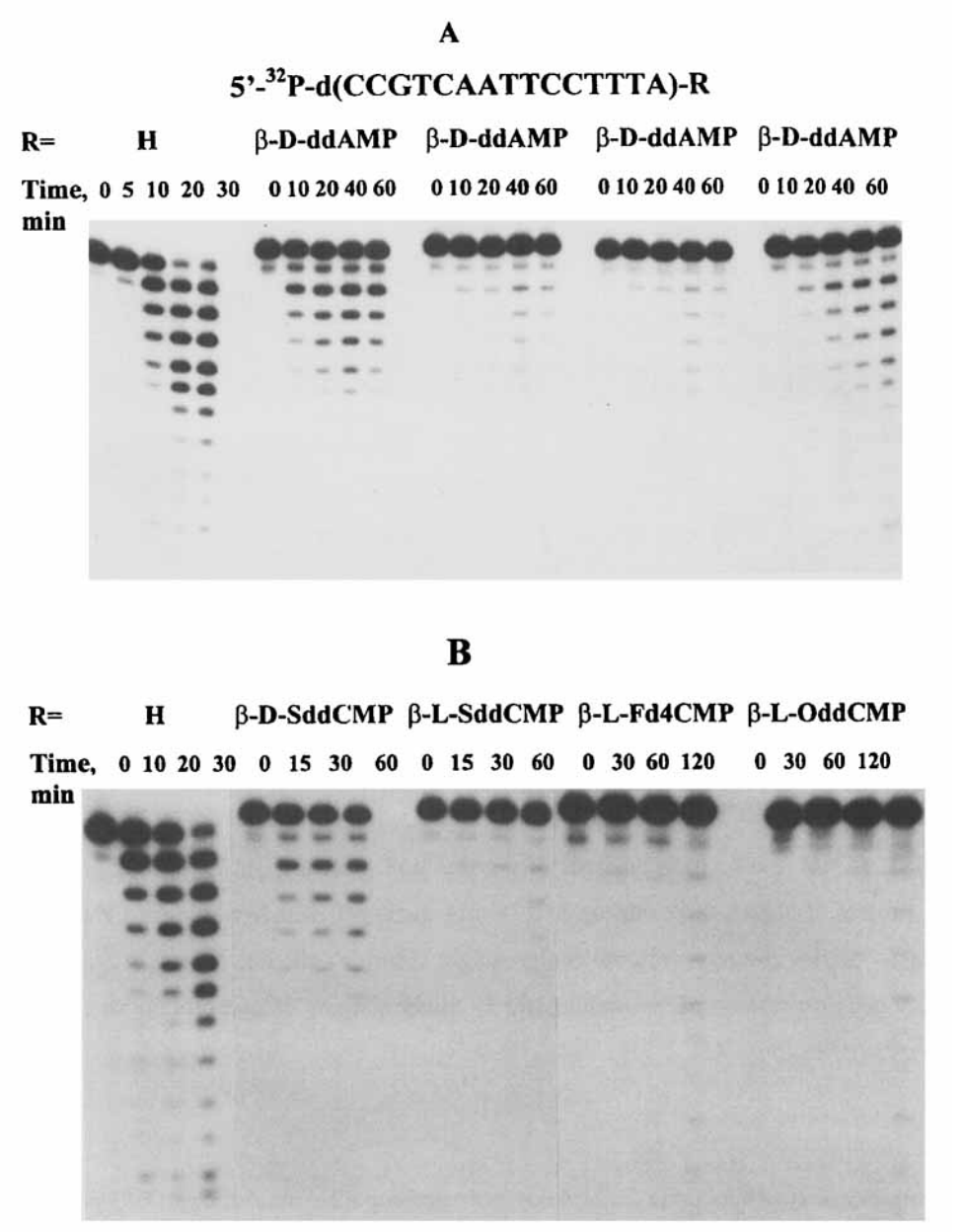

FIG. 2. A. Time-dependence of degradation of oligonucleotides terminated with β-D-ddNMP by the exonuclease of p53 protein under conditions described in Materials and Methods.

B. Time-dependence of degradation of oligonucleotides terminated with β-D- and β-L-nucleotide analogs by the exonuclease of p53 protein. Conditions are described in Materials and Methods. Degradation of the natural 15-mer is shown on the left (R=H).

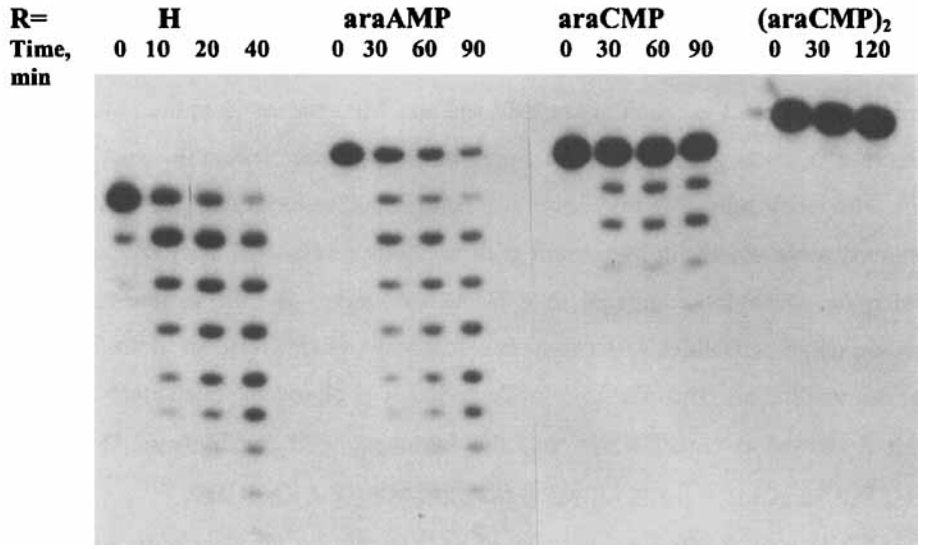

FIG. 3. Time-dependence of excision of araAMP, araCMP, and $(araCMP)_2$ from the 3'-end of oligonucleotide by the exonuclease of p53 protein. Conditions are described in Materials and Methods. Degradation of the natural 15-mer is shown on the left (R=H).

terminated with β-D-ddNMP compared with that of the natural 15-mer. The p53 protein could excise all four D-ddNMP residues, but rate of excision appeared to be different. While the natural 15-mer oligonucleotide was completely degraded in 20 min, substantial amounts of β-D-ddNMP-terminated primers were not digested. Among the β-D-ddNMP analogs examined, β-D-ddAMP at the 3'-end of primer, was preferred substrate for the exonuclease although the rate of excision was 10 fold less compared to that of natural dNMP. The efficiency of removal of β-D-ddGMP and β-D-ddTMP was almost equal on these two substrates, and less than that of β-D-ddAMP. Surprisingly, primer terminated with β-D-ddCMP was the poorest substrate for the exonuclease. FIG. 3 shows the time-dependence of degradation of DNA primers with β-D-SddCMP, β-L-SddCMP, β-L-Fd4CMP, and β-L-OddCMP at the 3'-end. The natural 15-mer and β-D-SddCMP terminated oligonucleotides were degraded by 90% and 40% in 30 min and 60 min, respectively. At the same time only 5-10% of the β-L-SddCMP residues could

be excised by the 3'-to-5' exonuclease activity of p53 protein in 60 min. β-L-Fd4CMP and β-L-OddCMP residues were very poor substrates for p53 protein. FIG. 4 shows the time-dependence of excision of araAMP and araCMP residues from the 3'-termini. The oligomer products generated indicate that the exonuclease cleaves the araAMP lesions with efficiency only 2-3 fold less than natural nucleotides. AraCMP residues were removed more slowly in agreement with previous results that adenosine analogs are preferable substrates compared to cytidine derivatives for the exonuclease of p53 protein. Oligonucleotides with two arabinofuranosylcytidine residues at the 3'-end were not degraded at all. The efficiency of degradation of oligodeoxynucleotides terminated with β-D- and β-L-nucleotides was the following: β-D-dNMP > araAMP > β-D-SddCMP >araCMP > β-L-SddCMP ≥ βL-Fd4CMP ≥ β-L-OddCMP.

Inhibition of exonuclease activity of p53 protein by oligonucleotide terminated with β-L-OddCMP. In order to compare the affinity of p53 protein for the primer terminated with a β-L-nucleotide, the effect of 15-mer primers with β-L-OddCMP at the 3'-end on the initial rate of 5'-[^{32}P]-15-mer primer degradation by exonuclease was measured as a function of the concentration of the β-L-OddCMP terminated primer. FIG. 4 shows that β-L-OddCMP terminated primers inhibit the degradation of 5'-[^{32}P] primer by 50% at concentrations 5 fold higher than the natural 15-mer primer. In these experiments the reaction conditions were chosen so that 50% inhibition of [^{32}P]-15-mer degradation by natural primer was observed when the ratio of their concentrations was equal 1:1.

DISCUSSION

The present view of the molecular action of tumor suppressor p53 protein implies a function as a cell-cycle regulator in response to DNA damage.[1-3] This protein also plays a critical role in the maintenance of genetic stability. Recent discovery of the intrinsic 3'-to-5' exonuclease activity of p53 protein provokes us to hypothesize that the 3'-to-5' exonuclease of p53 protein could play a role in the selectivity improvement of nucleoside analogs, used as anticancer or antiviral compounds. Because the incorporation of nucleoside analogs into the 3'-end of DNA is critical for their effect, their excision from DNA may decrease the chain termination potential and causes the reversibility of their action. The removal of nucleoside analogs may also decrease the

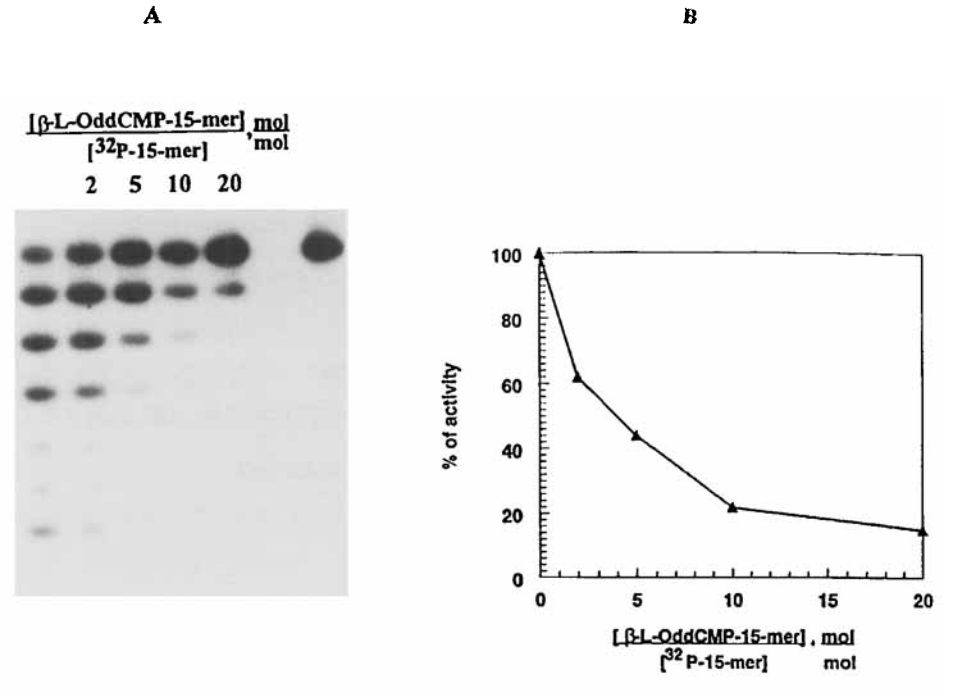

FIG. 4. A. Inhibition of 3'-to-5' exonuclease of p53 protein as a function of concentrations of β-L-OddCMP terminated primer. The concentration ratio of β-L-OddCMP terminated primer to that of 5'-[^{32}P]-15-mer primer is shown above the lanes. Left lane – degradation of [^{32}P]-primer in the absence of competitive primer; right lane – 15-mer primer taken into the experiments.

B. Quantitating analysis of the binding of p53 protein to the β-L-OddCMP terminated primer with the aid of densitometer (Molecular Dynamics). Reaction products are shown in *panel* A. Degradation of primer in the absence of competitive primer was taken as 100%. Reaction conditions as described under Material and Methods.

toxicity of anti-HIV/or anti-HBV nucleoside analogs, if they are incorporated into normal cellular DNA. At the same time tumor cells have often defective p53 protein.

In this study, we have shown the ability of p53 protein to remove a number of dideoxynucleoside analogs from the 3'-end of DNA. We selected therapeutically valuable compounds: araC is used for the treatment of leukemia;[11] araA was approved for the treatment of patients with herpes encephalitis;[12] β-L-SddC was recently

approved for the treatment of patients with HIV and/or HBV;[13] β-L-Fd4C was found to be one of the most effective anti-HBV compound;[14] β-L-OddC was shown to be effective against both solid and lymphoid tumors in *vivo*.[15,16] However, after the removal of drug, the amount of nucleoside residues in DNA decreased with time.[15] The exonuclease responsible for the excision of β-L-OddCMP, and some other terminators, is still unknown.

Here we have shown that the 3'-to-5' exonuclease activity of p53 protein is unlikely to be responsible for the excision of β-L-SddCMP, β-L-Fd4CMP, and β-L-O-ddCMP residues from DNA. In contrast to β-L-nucleotide analogs, all β-D-enantiomers tested so far appeared to be the subjects for excision by p53 protein. The present results indicate that araAMP and araCMP residues can be removed from DNA rather effectively (FIG.3). β-D-SddCMP was also a good substrate for exonuclease compared with its β-L-enantiomer (FIG. 2B). Thus, the 3'-to-5' exonucleose activity of p53 protein could play an important role in the removal of β-D-nucleoside analogs, once they were incorporated into DNA. Surprisingly, the excision of β-D-ddCMP from the 3'-termini of DNA proceeded at a much slower rate than that of other β-D-ddNMP (FIG. 2A) This suggests that differences in the nucleoside base may limit the rate of their excision by p53 protein. The most effective substrate among D-ddNMP residues was found to be β-D-ddAMP. The results obtained here also demonstrate that modification in the sugar residue of nucleotide analogs at the 3'-end of DNA plays a major role in the excision process. One would expect that exonuclease interacts with the 3'-end nucleotide of DNA, which might be critical for the functioning of the enzyme. Covalent linkage of β-L-nucleosides to the 3'-end of DNA are likely to induce changes in DNA structure that account for the inefficient excision of β-L-nucleotides from DNA through very slow phosphodiester bond hydrolysis or ineffective binding of enzyme to modified DNA. The structure of 1-β-D-arabinofuranosyl analogs has unlikely serious effect on the catalytic function of the enzyme and/or it's binding affinity for the template. Crystallographic and NMR studies[17-19] have shown that 1-β-D-arabinofuranose sugar does not significantly alter the conformation of nucleosides from those adopted by deoxynucleosides. Our studies have demonstrated that the difference in arabinofuranosyl nucleoside and deoxynucleoside structures does not significantly

affect the degradation of primers with araAMP compared to dAMP. The analysis of molecular conformations of β-D-OddC and β-D-ddC showed that while of β-D-OddC has a 3'-*endo* dioxolane ring conformation, β-D-ddC accommodates a 3'-*exo* conformation. Being a mirror image of the D-enantiomer, β-L-OddC should have 3'-*endo* conformation of dioxolane ring.[20] As was shown in this study, such modification has a dramatic effect on the ability of p53 protein to excise the analog from the 3'-end of DNA.

Since, β-D-nucleoside analogs incorporated at the DNA terminal are the substrates of p53 associated exonuclease, we hypothesized that the exonuclease might play an important role in the selectivity of nucleoside analogs against tumor defective in p53 protein, there by increasing their effect. Furthermore, after induction of p53 protein with nucleoside analogs, 3'-to-5' exonuclease could affect the action of β-D-nucleoside analogs against normal proliferating tissue by decreasing the amount of incorporated analog and subsequent toxicity.

Although β-L-nucleotide analogs are very poor substrates for the exonuclease activity of p53 protein, p53 protein binds to DNA terminated with β-L-OddCMP with an affinity only 5 fold lower compared with the natural primer. However, our analysis does not measure the precise position of p53 protein on the primer required for rapid catalysis. Besides the direct involvement of the exonuclease of p53 protein in the repair process of DNA terminated with β-L-nucleotides, the binding of p53 protein to terminated DNA may serve as a signal to induce the DNA repair process. Even though further studies are required to fully analyze the role of the 3'-to-5' exonuclease of p53 protein in the DNA repair, the differences in the expression of p53 protein in normal and cancer cells may play an important role in the activity of both β-D-and β-L-nucleoside analogs as anticancer compounds.

Acknowledgements. Supported by Grants from NIH CA-63477 and AI-42157
We would like to thank Dr. Karen Anderson of Yale University for her help with mass-spectrum analysis of p53 protein and very productive discussions.

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