The DNA double-stranded break repair protein endo-exonuclease as a therapeutic target for cancer

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Abstract

DNA repair mechanisms are crucial for the maintenance of genomic stability and are emerging as potential therapeutic targets for cancer. In this study, we report that the endo-exonuclease, a protein involved in the recombination repair process of the DNA double-stranded break pathway, is overexpressed in a variety of cancer cells and could represent an effective target for developing anticancer drugs. We identify a dicationic diarylfuran, pentamidine, which has been used clinically to treat opportunistic infections and is an inhibitor of the endo-exonuclease as determined by enzyme kinetic assay. In clonogenic and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays as well as in the *in vivo* Lewis lung carcinoma mouse tumor model, pentamidine is shown to possess the ability to selectively kill cancer cells. The LD_{50} of pentamidine on cancer cells maintained *in vitro* is correlated with the endo-exonuclease enzyme activity. Tumor cell that has been treated with pentamidine is reduced in the endo-exonuclease as compared with the untreated control. Furthermore, pentamidine synergistically potentiates the cytotoxic effect of DNA strand break and cross-link-inducing agents such as mitomycin C, etoposide, and cisplatin. In addition, we used the small interfering RNA for the mouse homologue of the endo-exonuclease to down-regulate the level of endo-exonuclease in the mouse myeloma cell line B16F10. Down-regulation of the endo-exonuclease sensitizes the cell to 5-fluorouracil. These studies suggested the endo-exonuclease enzyme as a novel potential therapeutic target for cancer. [Mol Cancer Ther 2004;3(8):911–9]

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Introduction

Cell cycle deregulation is a common denominator of cancer cells and has been the target for many of the current anticancer drugs (1, 2). Many anticancer chemotherapeutic agents inhibit DNA metabolism by targeting DNA replication process. Agents such as cisplatin induce DNA alteration in part by cross-linking DNA strands and inhibition of the subsequent enzymatic processing of the DNA-damaging sites by DNA repair enzymes (3–5). Other anticancer agents such as etoposide interfere with the topoisomerase enzymes, which modify DNA topology (6, 7). As a result, DNA breaks are introduced and become longer lived, resulting in cell death.

Living organisms repair DNA by a variety of mechanisms including excision repair (base excision and nucleotide excision) and DNA double-stranded break (DSB) repair (homologous recombination and nonhomologous end joining) systems (5, 8–12). These repair systems lessen the efficiency of cancer therapies that are dependent on chemotherapeutics, which target DNA. In addition, its enhanced capacity may allow a faster repair of spontaneous DNA lesions arisen from rapid proliferation of the cancer cells. On the other hand, a reduced capacity in DNA repair process has been reported to play a vital role in genomic instability and cancer development. This dual and opposing action of the DNA repair process indicated that, whereas DNA repair protein(s) could be a target for anticancer drug development, the choice of the DNA repair protein and its corresponding repair pathway as a target is highly critical. The ideal target should be the one that provides killing of the cancer cells but not enhancing mutagenesis in cancer cells. Among the DNA repair pathways, the homologous recombination process of the DNA DSB repair seems to best fit this criterion. Cells that are deficient in homologous recombination process are less prone to mutagenesis as cells lacking the DNA excision repair or the DNA mismatch repair pathway. An unrepaired DNA DSB is a lethal event (13, 14). As yet, however, there has not been a report in the literature on protein in this particular DNA repair process to be a target for anticancer drug development.

The enzyme endo-exonuclease has been shown to function in DNA DSB repair and recombination (15). The protein is highly conserved as antibody raised against the protein isolated from *Neurospora crassa* is immune reactive and is capable of inhibiting the enzymatic activity with the endo-exonuclease isolated from mammals including man (16–19). The molecular mass of the protein is 72 kDa (17, 20). The protein is expressed in high quantity during the growing phase of the cell cycle but not when cell reach confluence (18). The level of the protein is high in transformed cells (17). Overexpression of the yeast homologue on a multicopy plasmid results in an increase of cell survival following irradiation and an increase in

spontaneous and radiation-induced mitotic recombination between duplicated genes (21). In addition, transgene expression of the yeast homologue in mouse fibroblast cells increases homologous recombination of exogenous DNA (22). Molecular analysis of the endo-exonuclease indicates that the protein acts early in the DSB repair process (23). In this study, we provide two evidences that the endo-exonuclease be a good target for anticancer drug development. We identified a small molecule compound, pentamidine, which specifically inhibits the endo-exonuclease. Pentamidine shows anticancer activity, and synergistic activity with many of the chemotherapeutic agents target DNA both in cell lines and in a mouse tumor model. In addition, we showed that a knockdown of the endo-exonuclease with small interfering RNA (siRNA) potentiates the cancer cell killing effect of 5-fluorouracil (5-FU).

Materials and Methods

Cell Lines

The cell lines HT29, MCF7, HeLa, H520, H460, H661, and B16F10 were obtained from American Type Culture Collection (Manassas, VA). The M47 Lewis lung carcinoma cell line was kindly provided by Dr. P. Brodt (McGill University, Montreal, Quebec, Canada). The normal primary cell, NHDF, was obtained from Dr. Shirley Lehnert (McGill University). The cc531 cells were provided by Dr. Sandy Pang (University of Toronto, Toronto, Ontario, Canada). The cells were grown in RPMI supplemented with 10% FCS at 37°C in a humidified incubator with 5% CO_2.

Endo-exonuclease Levels

The endo-exonuclease level in the cell lines was determined with immunoblot method as described by Chow and Resnick (20). Exponentially growing cells were boiled in lysis buffer [0.125 mol/L Tris-HCl (pH 7.0), 20% glycerol, 4% SDS, 0.5 mmol/L EDTA]. The lysed cells were centrifuged at 10,000 × *g* for 10 minutes, and the supernatant (25 μL) was electrophoresed on a 10% SDS-PAGE gel according to the method described by Laemmli (24). Proteins that had been separated on the SDS-PAGE gel were transferred electrophoretically to a nitrocellulose membrane. The nitrocellulose membrane was treated with rabbit antiserum raised against the monkey CV-1 endo-exonuclease in buffer B [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 150 mmol/L NaCl] containing 0.5% skim milk powder according to the method described previously by Chow and Resnick (25). After the membrane had been washed three times in buffer B for 15 minutes, protein A conjugated with horseradish peroxidase in buffer B containing 0.5% skim milk powder was added to the membrane and incubated for 3 hours at room temperature. The membrane was subsequently washed with buffer B for 15 minutes. Positive signals were indicated by color development of the substrate 4-chloro-1-naphthol at the corresponding protein position in the horseradish peroxidase enzymatic reaction. Relative amount of positive signals was detected using a HP4c scanner and Light Tool Research software program.

Endo-exonuclease Isolation and Assay

The human endo-exonuclease was isolated according to the method described by Liu et al. (18). The cultured cells were detached with trypsin-EDTA, and the cell suspensions were centrifuged at 4°C with a speed of 700 × *g*. The cell pellets were washed twice with cold PBS. The cells were resuspended and sonicated in 20 mmol/L Tris-HCl (pH 7.5) containing 5 mmol/L EDTA and 1 mmol/L phenylmethylsulfonyl fluoride (buffer A). The resulting cell lysis suspensions were centrifuged at 4C at 10,000 × *g* for 15 minutes. The supernatants were loaded onto an antibody protein A-Sepharose affinity column as described previously (17, 20). After washing extensively with buffer A (i.e., until the $A_{280\ nm}$ of the eluates was 0), the column was eluted with buffer A containing 3.5 mol/L $MgCl_2$ to elute the endo-exonuclease. The eluted endo-exonuclease was dialyzed extensively against buffer A with at least two changes of buffer and one change of distilled water. The endo-exonuclease was concentrated by lyophilization.

The nuclease activities were determined by measuring the release of acid-soluble radioactivity from γ-[^{32}P]-labeled, heat-denatured single-strand pBR322 DNA according to the method described by Chow and Resnick (26). One unit of activity was defined as the amount of DNase that renders 1 μg DNA acid soluble in 30 minutes at 37°C. For the inhibition assay with the drugs, the drugs were added to the endo-exonuclease prior to the start of the nuclease reaction.

Clonogenic Assay

Clonogenic measurement of cell survival was used to determine the initial effectiveness of pentamidine according to method described in Sadekova et al. (27). In this method, log-phase cells (from 1,000 to 3,000 cells per 50 mm depending on plating efficiency) were seeded onto cell culture plates together with various drug concentrations (from 0.2 μmol/L to 20 mmol/L). After 1 week of growth, cell colonies were stained with crystal violet and the number of colonies (containing at least 16 cells) was counted.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method of determining cell growth/cytotoxicity offers a convenient alternative to determine cell survival. MTT is a tetrazolium salt cleaved by mitochondrial dehydrogenases of living cells. Cleavage converts yellow, water-soluble MTT to an insoluble, purple formazan crystal. The crystals can be solubilized with a 50% *N*,*N*-dimethylformamide (v/v), 20% SDS (w/v) solution (pH 4.7), and absorbance is determined at a wavelength of 570 nm. Dead cells will not cleave MTT, and uncleaved MTT is not detectable at this wavelength. The amount of MTT that is cleaved increases with increasing cell numbers and decreases as a result of cell cytotoxicity (28).

Cells were harvested from cell cultures using the standard protocol (trypsin-EDTA). The cells (1,000 to

5,000 cells depending on cell type in 5 μL) were plated and incubated overnight at 37°C before the addition of experimental reagents (i.e., the drug of interest; for the combination experiment, both drugs are added). After 2 days of incubation at 37°C, MTT (10 μL, 5 mg/mL) was added to all the experimental wells as well as the control medium well. The plates were further incubated for 4 hours. A MTT solubilization buffer (100 μL) was added and the plates were incubated overnight at 37°C. The plates were read on the ELISA plate reader with absorbance at 570 nm and reference at 630 nm.

siRNA Effect on Cell Survival

Plasmid-based siRNA against the mouse enzyme endo-exonuclease for gene knockdown was constructed using the GeneSuppressor System obtained from Imgenex Corp. (San Diego, CA). The DNA sequence of the siRNA is 5′-TCGATGTGAATTCCTGGTCGGAGTTGGAATTC-GAACTCCGACCAGGAATTCACATTTTT-3′. The cloning of the siRNA insert and transfection procedure were done according to the manufacturer's protocol. The transfected cells were treated with 5-FU with the MTT assay described above.

Lewis Lung Carcinoma *In vivo* Model

The Lewis lung carcinoma clone M47 is a metastatic model. These cells were maintained in RPMI 1640 supplemented with fetal bovine serum and penicillin-streptomycin. For tumor induction, cells were washed three times with phosphate buffer solution and resuspended at a dilution of 1×10^6 cells per 0.1 mL. Only cells with >95% viability were used for *in vivo* studies. The mouse strain used in this study is C57BL/10. After 1-week acclimatization, cells were transplanted s.c. as a suspension of tumor cells. All animals were inoculated at the same site. For the effect of drugs on the primary tumor, drug solutions were injected by i.p. every 2 days. Animals were subjected on a daily basis to general examination, and tumor growth was monitored over time.

To determine the effect of drugs on tumor metastasis, the tumors were allowed to reaches a size of 0.5 to 1.0 cm^2, mice were randomized into various groups, and drugs were given by i.p. At the end of each experiment, 16 days of treatment, animals were sacrificed and autopsied. Tumors, organs, or both were removed under sterile conditions. Lungs were fixed in Bouin's fixative, and lung surface metastases were counted using a stereomicroscope.

Combination Index Analysis

Synergistic interaction between pentamidine and chemotherapeutic agents was analyzed with the combination index method described by Chou et al. (29).

Blood Analysis

For some experiments involving drug combinations, blood was taken from three to five animals per group at the end of the treatment. Blood was collected in heparinized tubes and analyzed for hematologic values.

Statistical Analysis

The two-tailed Student's *t* test was used to compare statistical significance among various groups.

Results

The Level of Endo-exonuclease in Cancer Cells

The level of endo-exonuclease in various cell lines was determined by immunoblot method using the specific antibody raised against the monkey CV-1 endo-exonuclease. The levels of endo-exonuclease in cancer cells are 30- to 80-fold higher than in the NHDF normal primary cell (Fig. 1 and Table 1).

Inhibition of the Endo-exonuclease Activity

It has been founded previously that the endo-exonuclease in *Pneumocystis carinii* is inhibited by some dicationic diarylfurans (19). We analyzed a series of small molecules of this class of chemicals and identified pentamidine as a specific inhibitor of the human endo-exonuclease (Table 2). To show that the inhibition of the dicationic diarylfurans is capable to act directly on the endo-exonuclease protein rather than due to the DNA binding interference of the enzymatic activity, we used the ssDNA as the substrate for the analysis. Pentamidine is capable of binding to the minor groove of dsDNA but not to ssDNA (30–32). The inhibition of the endo-exonuclease activity by pentamidine seems to be specific and is not due to nonspecific inhibition of any nuclease. In this case, we use the S1 nuclease, a nuclease that has activity with ssDNA, as a reference enzyme for comparison. Pentamidine does not inhibit S1 nuclease activity (Fig. 2A). We have also analyzed two compounds, distamycin A and berenil, that belonged to the same class of compound as pentamidine. Whereas distamycin A seems to show some inhibition of the endo-exonuclease activity, berenil is not effective at all. In addition, specific DNA minor groove binding agents such as mitomycin C or the topoisomerase inhibitor etoposide does not inhibit the endo-exonuclease (Table 2). The inhibition of pentamidine on the endo-exonuclease seems to act directly on the enzyme. An addition of the ssDNA substrate during the enzymatic reaction did not alter the inhibition kinetic (Fig. 2B).

Cell Survival in the Presence of Pentamidine Using Clonogenic Assay or MTT Assay

Clonogenic measurement of cell survival in the presence of pentamidine of NHDF primary cells, MCF7 cells, and HeLa cells is shown in Fig. 2C. These three cell lines were

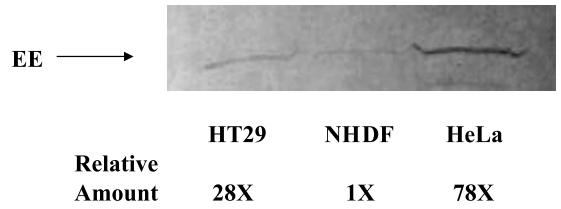

Figure 1. Relative amount of endo-exonuclease in cancer cell lines. Cell extracts from cancer cell lines HT29, HeLa, and the primary cell line NHDF were analyzed for the endo-exonuclease level with immunoblot method as described in Materials and Methods. Protein (50 μg) was loaded onto each lane. Density of each band was determined and the relative amount of endo-exonuclease in cancer cell lines is calculated by considering the level of endo-exonuclease in NHDF as 1.

Table 1. Relative amount of endo-exonuclease and the LD_{50} dose of pentamidine in cell lines

Cell Line	Relative Amount of Endo-exonuclease	LD_{50} (μmol/L)*±SD
NHDF	1	>1,000
HT29	28	270 ± 3
MCF7	32	150 ± 1
Colo-205	36	5.9 ± 0.3
MDA-MB-435	56	2.4 ± 0.07
DA3	58.8	2.4 ±0.06
B16F10	75.6	1.2 ± 0.05
Lewis lung carcinoma	75.6	1.2 ± 0.06
HeLa	78.6	NA

*Determined with MTT assay. The SD is calculated from five assays.

chosen as these represent the different level of endo-exonuclease in the cell and their sensitivity to pentamidine treatment. The results indicate that pentamidine preferentially attacks cancer cells in a dose-dependent manner.

The effect of pentamidine on cell survival of several cancer cell lines (H520, H460, H661, MCF7, and HT29) was analyzed with the MTT method. The LC_{50} of the cell survival obtained with exposure to pentamidine in a 2-day and 4-day MTT assay is reported in Table 3. The effectiveness of pentamidine on cancer cell killing is similar to the effect of mitomycin C, etoposide, or cisplatin. The killing effectiveness of pentamidine is correlated with the relative level of the intracellular endo-exonuclease (Table 1). Furthermore, the treatment of cc531 rat colon carcinoma cells with pentamidine has lower endo-exonuclease level when compared with the untreated control (Fig. 2D).

The effect of distamycin A and berenil on cell survival of several cancer cell lines was also analyzed and the result was compared with pentamidine. Whereas both distamycin A and berenil are not effective in killing the cancer cells, distamycin A seems to be more potent than berenil (Table 4).

Table 2. Inhibition of purified human endo-exonuclease activity by chemotherapeutic agents

Chemotherapeutic Agent	% Inhibition ± SE
Pentamidine (25 μmol/L)	37 ± 7
Pentamidine (50 μmol/L)	50 ± 5
Pentamidine (100 μmol/L)	100
Distamycin A (38 μmol/L)	30 ± 4
Berenil (2 mmol/L)	17 ± 4
Mitomycin C (50 μmol/L)	0
Etoposide (VP-16; 50 μmol/L)	0

NOTE: Values were determined from three assays. Mitomycin C is a DNA alkylating agent and a DNA minor groove binder. Etoposide (VP-16) is a topoisomerase inhibitor. Both agents are currently used in the treatment of cancer.

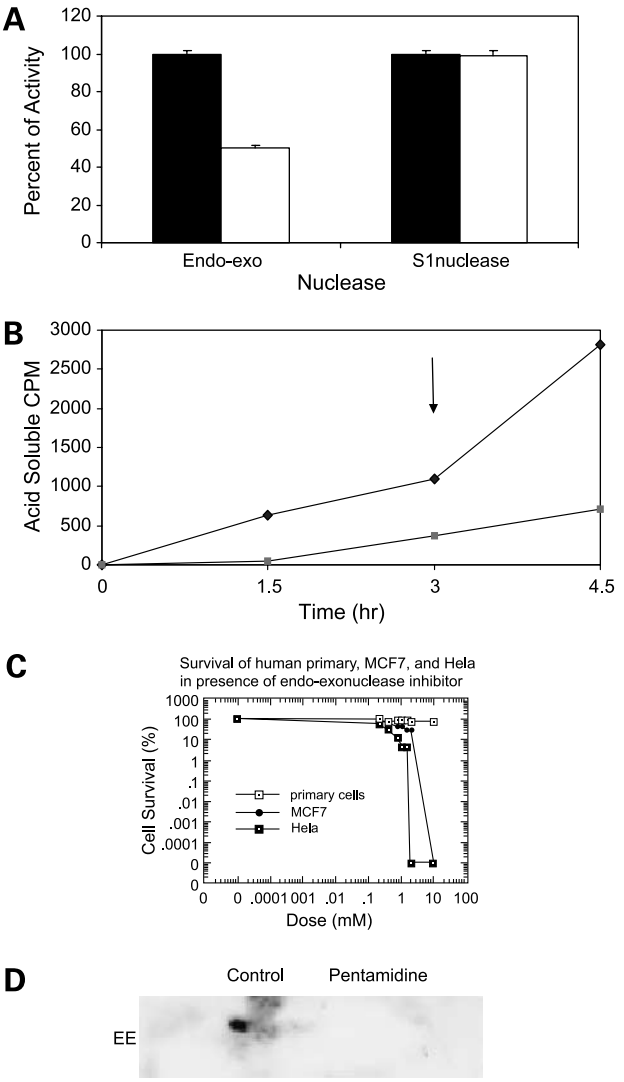

Figure 2. The effect of pentamidine. **A,** inhibition of purified human endo-exonuclease and S1 nuclease by 50 μmol/L pentamidine. DNase activity in the presence of 50 μmol/L pentamidine was measured as described in Materials and Methods. Three independent ssDNA assays: ssDNA activity in the absence of pentamidine (■) and ssDNA activity in the presence of 50 μmol/L pentamidine (□); *bars,* SE. **B,** enzyme kinetic of the endo-exonuclease. The effect of an addition of 50-fold DNA substrate during enzymatic reaction of the purified human endo-exonuclease was measured in the absence (♦) and presence (□) of 50 μmol/L pentamidine. The excess DNA substrate was added at 3 hours into the reaction time point (*arrow*). If the inhibitory effect of the pentamidine is due to its effect in intercalating the DNA substrate, then the addition of the excess amount of DNA substrate to the reaction mixture would reduced the inhibitory effect of pentamidine and the release of acid soluble CPM should increased. On the other hand, if pentamidine is acting directly on the endo-exonuclease, the addition of excess amount of substrate would not alter the inhibitory effect of pentamidine. **C,** the effect of pentamidine on cell survival as determined by clonogenic assay of NHDF primary cells, MCF7 cells, and HeLa cells were determined by clonogenic assay. Pentamidine has a LD_{50} (±SD) with HeLa cells at 0.15 ± 0.01 mmol/L and a LD_{50} with MCF7 at 0.25 ± 0.01 mmol/L. Three assays were done. **D,** the effect of pentamidine treatment on the level of enzyme endo-exonuclease (*EE*) in cc531 colon carcinoma cells. Protein (20 μg) from cell lysate was loaded onto the slot using the Bio-Rad slot blot apparatus. The endo-exonuclease was detected by immunoblot as described in Materials and Methods.

Combination of Endo-exonuclease Inhibitor and DNA Break Inducers

The effect of combining pentamidine with mitomycin C, etoposide, and cisplatin is shown in Table 5. The effect of pentamidine with sublethal concentrations of mitomycin C, etoposide, or cisplatin greatly increases the killing of the cancer cells, indicating a synergistic of action.

The Effect of siRNA of the Endo-exonuclease on Cell Survival

The effect of siRNA against the mouse endo-exonuclease on 5-FU sensitivity in the mouse B16F10 melanoma cell is reported in Fig. 3. The treatment of siRNA against the mouse endo-exonuclease reduces the amount of endo-exonuclease as compared with untreated or transfection agent treated control. The specificity of the siRNA is shown by the level of α-tubulin, which is not affected by the siRNA treatment (Fig. 3B). The transfection of siRNA shows a specific reduction of the cell viability as evidenced by comparison of control cells and those treated with the transfection reagent alone (Fig. 3A). The siRNA-treated cells has ~50% to 55% cell viability of the control and the transfection reagent treated cells. The cell viability between the control and the transfection reagent treated cells is the same.

5-FU, an antimetabolite drug, works by inhibiting essential biosynthetic processes or by being incorporated into macromolecules such as DNA and RNA and inhibiting their normal function (for a review, see ref. 33). 5-FU is converted intracellularly to several active metabolites (fluoro-dUMP, fluoro-dUTP, and fluoro-UTP). These active metabolites disrupt RNA synthesis and the action of thymidylate synthase, resulting in a severely disrupted DNA synthesis and repair that produce lethal DNA damage. In addition, 5-FU has been used for >40 years in the treatment of cancer. The result of siRNA on the endo-exonuclease in the presence of 5-FU, in conjunction with the MTT results (Table 5), would provide some insight into the range of potential chemotherapeutic agents that targets the DNA to be in synergy with endo-exonuclease inhibition. The siRNA-treated cells are highly sensitivity to the DNA-damaging agent 5-FU (Fig. 3A). Student's t test shows that the difference of cell survival in control versus siRNA is highly significant ($P < 0.0003$). Likewise, the cell survival between cells treated with transfection agent only and cells treated with siRNA is also significant ($P < 0.0002$). There is no statistical difference between control and cells that were treated with transfection agent only ($P = 0.15$).

Tumor Model

We analyzed the effect of pentamidine using the Lewis lung tumor model after 16 days of treatment. The effect of pentamidine on tumor growth is dose dependent. Pentamidine at 25 and 50 mg/kg inhibits tumor growth by 20% and 60%, respectively. The level of tumor growth inhibition by pentamidine at these two concentrations is similar to the treatment of cisplatin at 1 and 3 mg/kg (Fig. 4A). Furthermore, the two compounds of pentamidine (50 mg/kg) and cisplatin (3 mg/kg) can be given in combination with no increase of serious side effects. The hematologic values between pentamidine-treated and untreated mice are similar (Table 6). The combined treatment of pentamidine and cisplatin does not seem to decrease the value of WBC. Treatment with a combination of pentamidine and cisplatin results in a synergistic therapeutic efficacy that is better than the treatment with either pentamidine or cisplatin alone (Fig. 3A). Furthermore, three mice in this group showed no sign of tumor at the end of the experiment (Fig. 3B). Using the combination index analysis, the combination index obtained for the combine effect of pentamidine and cisplatin is 0.69. The result suggests a synergistic action between these two compounds. Similar results were also obtained with pentamidine in combination with Adriamycin (data not shown).

We have also analyzed the effect of pentamidine on tumor metastases using the Lewis lung tumor model. Pentamidine reduces lung metastases in a dose-dependent manner (10 mg/kg pentamidine by 22%, 25 mg/kg pentamidine by 25%, and 50 mg/kg pentamidine by 49%). In addition, pentamidine at 50 mg/kg is as effective

Table 3. Comparison of the LC_{50} of various anticancer agents on cancer cell lines with MTT assay

Cancer Cell Type	Pentamidine (mmol/L) ± SD		Mitomycin C (mmol/L) ± SD		Etoposide (mmol/L) ±SD		Cisplatin (mmol/L) ±SD	
	2 d	4 d	2 d	4 d	2 d	4 d	2 d	4 d
H520	0.24 ± 0.01	0.13 ± 0.02	0.234 ± 0.026	0.130 ± 0.018	>34	>34	0.50*	—
H460	1.34 ± 0.08	0.16 ± 0.01	0.065 ± 0.011	0.030 ± 0.002	>34	>34	0.50*	—
H661	0.15 ± 0.05	0.07 ± 0.01	0.006 ± 0.001	0.008 ± 0.001	28	15.6 ± 0.7	0.41*	—
MCF7	0.15 ± 0.01	0.08 ± 0.01	0.034 ± 0.016 0.024*	0.013 ± 0.001	1 ± 0.13 1*	1.1 ± 0.14	0.49*	—
HT29	0.27 ± 0.03	0.06 ± 0.01	0.008 ± 0.001 0.024*	0.008 ± 0.001	0.7 ± 0.04 0.7*	0.4 ± 0.02	0.48*	—

NOTE: The cancer cell types are H520-NSCLC (squamous carcinoma, primary tumor), H460-NSCLC (large cell carcinoma, pleural effusion), H661-NSCLC (large cell carcinoma, lymph node), MCF7-breast cancer (adenocarcinoma, pleural effusion), HT29-colon cancer (adenocarcinoma, primary tumor). The length of time that the cells are exposed to the compound: 2 and 4 days.
*Data obtained from the National Cancer Institute.

Table 4. Comparison of the LC_{50} of pentamidine, distamycin A, and berenil on cancer cell lines with MTT assay

Cancer Cell Type	Pentamidine (mmol/L) ± SD	Distamycin A (mmol/L) ± SD	Berenil (mmol/L) ± SD
H520	0.24 ± 0.01	>2.0	>4.0
H460	1.34 ± 0.08	>2.0	>4.0
H661	0.15 ± 0.05	>2.0	>4.0
MCF7	0.15 ± 0.01	1.52 ± 0.03	3.0 ± 0.1
HT29	0.27 ± 0.03	>2.0	>4.0

NOTE: The cancer cell types are H520-NSCLC (squamous carcinoma, primary tumor), H460-NSCLC (large cell carcinoma, pleural effusion), H661-NSCLC (large cell carcinoma, lymph node), MCF7-breast cancer (adenocarcinoma, pleural effusion), HT29-colon cancer (adenocarcinoma, primary tumor). The length of time that the cells are exposed to the compound: 2 days.

as 4 mg/kg cisplatin. The combination of 50 mg/kg pentamidine and 4 mg/kg cisplatin produces the best reduction in lung metastases (>70%) than treatment with either pentamidine or cisplatin alone (data not shown).

Discussion

The endo-exonuclease is an enzyme that possesses 5′-3′ exonucleolytic activity with dsDNA and endonucleolytic activity with ssDNA (17, 20, 34, 35). It acts early in the repair of DNA DSB (23). The expression of the endo-exonuclease in cancer cells is much higher than in normal primary cells (Fig. 1 and Table 1) and may be related to the rapid growth of the cancer cell. The expression of the endo-exonuclease is high during the fast-growing phase of the cell cycle and is expressed at very low levels at the stationary phase of the cell cycle (17, 18, 25, 36). In addition, the biological activity of the endo-exonuclease in transfected mammalian cells is shown by an increase in nuclease activity and increased resistance to DNA-damaging agents as well as elevated levels of recombination capacity (22, 37). These unique features of the endo-exonuclease suggest that it might be a target for drug development for the homologous recombination pathway of the DNA DSB repair and cancer. In a search for an inhibitor of the endo-exonuclease, we discovered that the small molecular compound pentamidine possesses inhibiting activity on the enzyme in a concentration-dependent manner and with

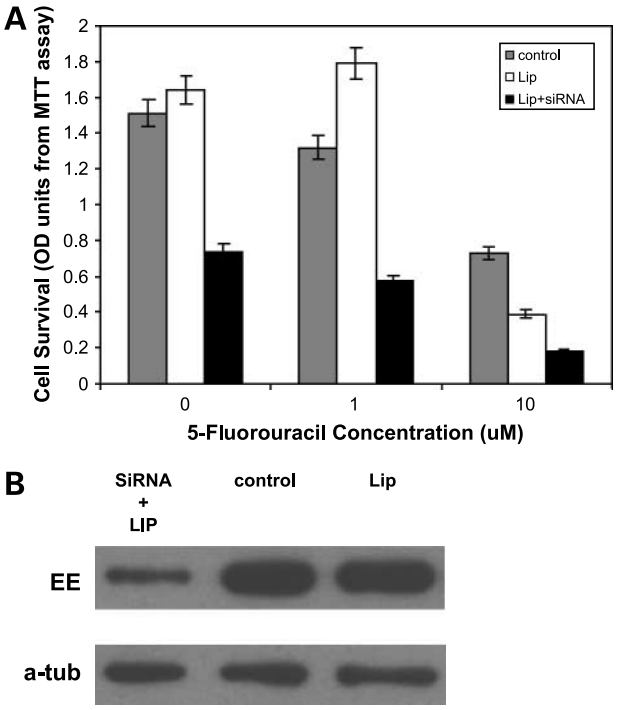

Figure 3. The effect of siRNA targets the endo-exonuclease on cell viability and on 5-FU sensitivity. **A,** 5-FU sensitivity of B16F10 cells from three experiments [control (□), transfection agent (□), and siRNA (■)]; *bars,* SE. **B,** the level of enzyme endo-exonuclease was determined by Western blot. α-Tubulin (*a-tub*) was used as the internal control for protein loading.

specificity (Fig. 2 and Tables 1 and 2). In addition, *in vitro* inhibitory activity of the dicationic diarylfurans seems to be correlated to the *in vivo* killing of the cells with the MTT assay (Tables 2 and 4). Furthermore, because the killing of the cells by inhibiting the endo-exonuclease is due to the inability of the cell to repair the DNA breaks arisen from cell growth, a longer exposure time of the cell to the endo-exonuclease inhibitor would result in a greater efficacy of cell killing. A situation of which is reflected in the MTT assay of 2 and 4 days of drug exposure in the various cell lines (Table 3).

Pentamidine belongs to a general group of compounds known as dicationic diarylfurans. Dicationic diarylfurans

Table 5. LC_{50} of pentamidine on cancer cells when used alone or in combination with other anticancer agents

Cancer Cell Type	Pentamidine (mmol/L) ± SD	Pentamidine (mmol/L) with Mitomycin C (1.56 μmol/L) ±SD	Pentamidine (mmol/L) with Etoposide (34 μmol/L) ±SD	Pentamidine (mmol/L) with Cisplatin (0.025 μmol/L) ±SD
H661	0.15 ± 0.05	0.0029 ± 0.0006	0.10 ± 0.016	0.039 ± 0.005
MCF7	0.15 ± 0.01	0.0029 ± 0.0003	0.049 ± 0.004	0.082 ± 0.005
HT29	0.27 ± 0.03	0.0022 ± 0.0006	0.085 ± 0.013	0.032 ± 0.004

NOTE: Length of exposure to mixture is 2 days. The concentration of mitomycin C (1.56 μmol/L), etoposide (34 μmol/L), and cisplatin (0.025 μmol/L) gives <5% of cell killing.

and dicationic carbazoles are under development as therapeutic agents against opportunistic infections. Whereas their ability to bind to the minor groove of DNA has been established, the complete mechanism of action is not known. In an attempt to evaluate the mechanism of action of pentamidine, Bornstein and Yarbro (38) showed that at 4 mg/mL pentamidine inhibits the *in vitro* incorporation of [^{3}H]thymidine (79%), [^{3}H]uridine (64%), [^{3}H]-algal protein hydrolysate (39%), and [^{32}P]-orthophosphate (12%) in 6C3HED tumor ascites of C3H mice. At 0.5 mg/mL, however, the inhibition of incorporation is marginal (2%, 0%, 0%, and 13%, respectively). Furthermore, the therapeutic activity of the dicationic diarylfurans against opportunistic infections does not correlate to the ability of the compound to bind to the minor groove of DNA (39), suggesting that the mechanism of action of this class of chemicals may involve specific targets other than the DNA. Indeed, Hildebrandt et al. (19) showed that the molecular target for some of the diarylfurans, diarylfuran and 2,5-bis[4-(*N*-isopropylguanyl)phenyl]furan, in *P. carinii*, *Cryptococcus neoformans*, *Candida albicans*, and *Saccharomyces cerevisiae* is the endo-exonuclease. In addition, we analyzed two other compounds in the similar class as pentamidine. They are distamycin A and berenil. The ability of these compounds to inhibit cancer cell growth using the MTT method seems to correlated to the ability of the compound to inhibit the endo-exonuclease activity (Tables 2 and 4). On the other hand, specific DNA minor groove binder and intercalating agent mitomycin C does not show any endo-exonuclease inhibitory activity (Table 2). Using the cc531 rat colon carcinoma cells, the treatment of the tumor cells with pentamidine results in a lowering of the endo-exonuclease level as compared with the corresponding untreated control (Fig. 2D).

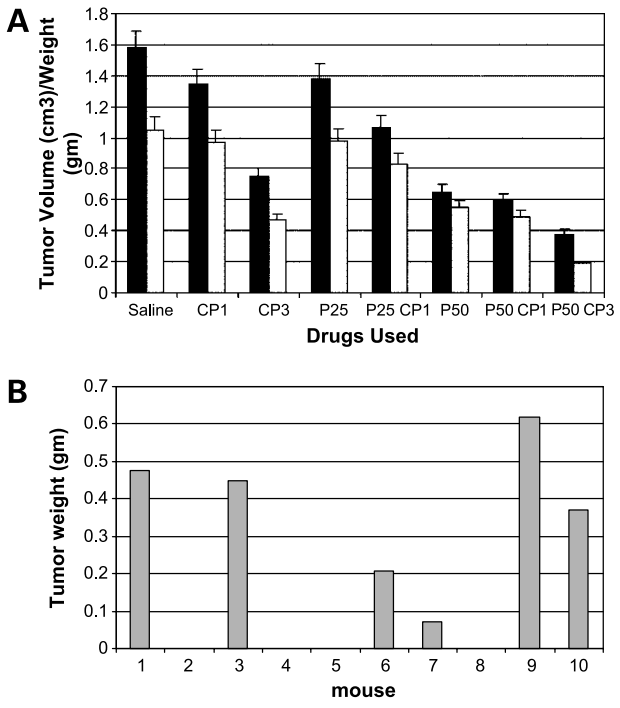

Figure 4. The effect of pentamidine alone and in combination with cisplatin on tumor growth in the Lewis lung carcinoma mouse tumor model. Ten mice were tested in each group. **A,** the drug was given every second day for 2 weeks at the dosages indicated according to the schedule described in Materials and Methods. *CP1,* cisplatin (1 mg/kg); *CP3,* cisplatin (3 mg/kg); *P25,* pentamidine (25 mg/kg); *P25 CP1,* pentamidine (25 mg/kg) + cisplatin (1 mg/kg); *P50,* pentamidine (50 mg/kg); *P50 CP1,* pentamidine (50 mg/kg) + cisplatin (1 mg/kg); *P50 CP3,* pentamidine (50 mg/kg) + cisplatin (3 mg/kg). Tumor volume (*black columns*) and tumor weight (*gray columns*) within the group of mice; *bars,* SD. **B,** tumor from each of the mice treated with the combination of pentamidine (50 mg/kg) and cisplatin (3 mg/kg) was surgically removed at the end of the experiment. The tumor weight from each of the mice is also presented.

Table 6. The effect of cisplatin, pentamidine, and combination on WBC

Treatment	WBC Count ± SD (10^9/L)
Saline	3.3 ± 0.5
Cisplatin (3 mg/kg)	2.3 ± 0.4
Pentamidine (10 mg/kg)	2.6 ± 0.3
Pentamidine (25 mg/kg)	3.3 ± 0.6
Pentamidine (50 mg/kg)	2.8 ± 0.5
Pentamidine (10 mg/kg) + cisplatin (3 mg/kg)	2.9 ± 0.2
Pentamidine (25 mg/kg) + cisplatin (3 mg/kg)	3.3 ± 0.7
Pentamidine (50 mg/kg) + cisplatin (3 mg/kg)	3.0 ± 0.7

NOTE: Blood was taken from three to five animals per group at the end of the treatment. WBC is expressed as the indicated number × 10^9/L.

Recently, Pathak et al. (40) reported that pentamidine is found to inhibit the oncogenic PRL family PTPases and has *in vitro* growth inhibitory activity against human cancer cell lines that express the endogenous PRLs. Pentamidine given at a tolerable dose markedly inhibited the growth of WM9 human melanoma tumors in nude mice. We do not, however, believe that these results contradicted our hypothesis the endo-exonuclease is a target for pentamidine. Because we do not have the X-ray crystal structure for the endo-exonuclease, we could only speculate the possible underlying possibility of the two findings. The MRE11 protein, which is a DNA repair nuclease having similar enzymatic characteristics as the endo-exonuclease in this study, contains a phosphatase-like, dimanganese binding domain capped by a unique domain controlling active site access (41). The action of pentamidine with PRLs may be due to the similar structure of the active site between the PRLs and the endo-exonuclease. Given the inhibitory effect of pentamidine on PRLs is immediate but requires a drug exposure of 6 days to determine the growth inhibitory activity in the MTT assay (40), the result suggests that cell death is likely due to an indirect consequence of the pentamidine treatment. Likewise, our result from the 2-day and 4-day exposure time of the cancer cells to pentamidine supports an indirect role of pentamidine in cell growth inhibitory activity (Table 3). The 4-day exposure to pentamidine increases cell death in all the cell types studied

than the 2-day exposure. In contrast, cell killing by a direct cytotoxic agent such as mitomycin C does not increase cell death with all the cell types examined when the MTT assay is carried over from a 2-day exposure to a 4-day exposure (Table 3). The inhibition of the endo-exonuclease by pentamidine will not produce a direct killing of the cancer cell. Cell death occurs because the inability of the cell to repair the DNA breaks arisen from cell growth. Longer exposure time of the cell to pentamidine would result in a greater accumulation of DNA damages (hence, an increase of cell death). The knockdown experiment of the siRNA against endo-exonuclease presented in Fig. 3 supports that the endo-exonuclease itself is a target for anticancer activity (see below). Perhaps antisense oligonucleotide or siRNA against the PRLs would clarify whether the PRLs are targets for anticancer development.

One prediction as a consequence of inhibition of DNA DSB mechanism is cell hypersensitivity to DNA break-inducing agents. This is supported by the fact that the inhibition of the endo-exonuclease sensitizes the cancer cells to chemotherapeutic agents such as cisplatin, mitomycin C, and etoposide. The synergistic effect of pentamidine in combination with various DNA strand-break agents is highly effective in killing cancer cells *in vitro* (Table 5). This synergy of action between pentamidine and cisplatin to inhibit tumor growth is likewise observed in an *in vivo* mouse tumor model (Fig. 4). In the group of mice that has been treated with pentamidine and cisplatin, three of the mouse showed no sign of the tumor at the end of the experiment.

The specificity of the pentamidine on the endo-exonuclease *in vivo* is also reflected by the hematologic results between the pentamidine-treated mice and the control. The treatment of pentamidine did not produce any hematologic alteration typical for DNA-specific chemotherapeutic agents (Table 6). Our results also suggest that pentamidine inhibits specifically the homologous recombination aspect and not the nonhomologous end joining aspect of the DNA DSB pathway. Because the nonhomologous end joining aspect of the DNA DSB repair process is required for immunoglobulin V(D)J switching, its inhibition would produce neutropenia in treated mice, a phenomenon that is not observed (42).

The validity of the endo-exonuclease to be a target for anticancer drug development is confirmed with the knockdown of the endo-exonuclease using the siRNA strategy (43, 44). In an agreement with the MTT results with the inhibition of the endo-exonuclease by pentamidine (Table 3), the knockdown of endo-exonuclease has a lower cell viability and is hypersensitized to the killing by DNA-damaging agents (Fig. 3 and Table 5).

In conclusion, we report the first evidence that the endo-exonuclease of the DNA DSB repair pathway is a target for anticancer drug development. Endo-exonuclease inhibition affects tumor growth with an efficacy similar to many chemotherapeutic agents targeting the DNA but with a reduced side effect. The result opens a new target for new drug discovery in the field of cancer therapy.

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