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Novel Cytoprotective Inhibitors for Apoptotic Endonuclease G

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Apoptotic endonuclease G (EndoG) is responsible for DNA fragmentation both during and after cell death. Previous studies demonstrated that genetic inactivation of EndoG is cytoprotective against various pro-apoptotic stimuli; however, specific inhibitors for EndoG are not available. In this study, we have developed a high-throughput screening assay for EndoG and have used it to screen a chemical library. The screening resulted in the identification of two potent EndoG inhibitors, PNR-3-80 and PNR-3-82, which are thiobarbiturate analogs. As determined by their IC_{50}s, the inhibitors are more potent than $ZnCl_2$ or EDTA. They inhibit EndoG at one or two orders of magnitude greater than another apoptotic endonuclease, DNase I, and do not inhibit the other five tested cell death-related enzymes: DNase II, RNase A, proteinase, lactate dehydrogenase, and superoxide dismutase 1. Exposure of natural EndoG-expressing 22Rv1 or EndoG-overexpressing PC3 cells rendered them significantly resistant to Cisplatin and Docetaxel, respectively. These novel EndoG inhibitors have the potential to be utilized for amelioration of cell injuries in which participation of EndoG is essential.

Introduction

Any type of cell death is characterized by nuclear DNA fragmentation, which is a limiting step and necessary mechanism of cell death, as after DNA fragmentation, cell death becomes irreversible (Hengartner, 2001). Cellular DNA fragmentation is catalyzed by apoptotic endonucleases. One of the most active representatives of this group is endonuclease G (EndoG). Other apoptotic endonucleases include DNase I, DNase II, their homologues, and caspase-activated DNase. EndoG is the only apoptotic endonuclease, which is a nuclease (DNase/RNase) and that is located in mitochondria (Cote and Ruiz-Carrillo, 1993, Parrish *et al.*, 2001).

The enzyme was first described by Ruiz-Carrillo and Renaud (1987) as an endonuclease with a unique site selectivity, initially attacking poly(dG).poly(dC) sequences in double-stranded DNA, from which the enzyme's name was derived. EndoG is synthesized as an inactive 33-kDa precursor. Mature 28-kDa enzyme produced by cleavage of signal peptide translocates to the mitochondrial intermembrane space, where it forms an active homodimer. During apoptosis, EndoG translocates to the nucleus where it cleaves DNA without notable sequence specificity (Cote *et al.*, 1989).

Similar to other apoptotic endonucleases, overexpression of EndoG is highly cytotoxic, while overexpression of inactivated EndoG does not induce cell death (Schafer *et al.*, 2004). More physiologically relevant are observations of the increased sensitivity to cytotoxic insults in cells expressing EndoG compared with EndoG-deficient cells (Basnakian *et al.*, 2006; Wang *et al.*, 2008). Genetic knockout or knockdown of EndoG in mice or cells is harmless for normal cellular function, but ameliorates injurious insults (Apostolov *et al.*, 2007). Thus, an inhibition or silencing of EndoG could potentially be used for tissue protection during injury and disease. However, the complete absence of clinically applicable EndoG inhibitors presents an obstacle to this therapeutic strategy.

One of the most effective methods for the identification of new small-molecule inhibitors is screening of chemical libraries. For this, the availability of a high-throughput screening (HTS) assay is crucial. Several available current assays for endonuclease activity, such as plasmid incision assay (PIA) (Basnakian *et al.*, 2002), random oligonucleotide-primed synthesis assay (ROPSA) (Basnakian and James, 1996), radial diffusion assay (Takeshita *et al.*, 2000), and zymogram gel electrophoresis (Basnakian *et al.*, 2005), are time consuming and labor intensive. In addition, they are often nonsensitive, nonspecific, or nonquantitative. Thus, the use of any of these assays for HTS applications is problematic.

In an attempt to set up an efficient screening of an in-house chemical library, we started with the development of a new HTS assay for EndoG. The assay utilizes a self-quenched fluorescent reporter DNA probe. This study resulted in the identification of two specific inhibitors of EndoG, which are also cytoprotective against *in vitro* cell injury.

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Materials and Methods

Library of compounds

The chemical library used in this study contained 1040 compounds, all of which were small molecules (≤500 MW) designed as drug-like compounds for oral delivery, and thoroughly characterized with regard to structure, purity, and physicochemical properties. All compounds were initially dissolved in HPLC-grade dimethylsulfoxide (DMSO) to afford a 10-mM stock solution. The solutions were loaded into 96-well master plates (Thermo, Rochester, NY), at 80 compounds per plate. The solutions in each master plate were then diluted with DMSO to afford the serial dilution rate, for example, 1–0.1 mM. These solutions were used as 100× stocks so that the final concentration of DMSO in the reaction mixture was 1%.

Recombinant EndoG purification

Mature mouse or human *EndoG* gene was inserted in pET29b vector and expressed in *Escherichia coli* BL21 (DE3). The histidine-tagged recombinant EndoG (recEndoG) was obtained by purification of *E. coli* protein extract through HisTrap FF crude column (Amersham Bioscience, Piscataway, NJ) followed by PD-10 column desalting, concentration, and storage in 50% glycerol at −20°C. The activity of the resulting enzyme preparation and the molecular mass of the enzyme were determined using zymogram gel electrophoresis performed as previously described (Basnakian *et al*., 2002).

High-throughput EndoG screening assay

A reaction mixture was prepared in white 96-well plates (Costar, Corning, NY) as follows: 0.25 μM endonuclease activity probe (Cy5.5-labeled oligonucleotide described in U.S. provisional patent Serial No. 61/716,097 filed 10/19/12), 0.1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 1 μL of test compound solution in DMSO, and nuclease-free water to a total volume of 100 μL. The background (negative control) and uninhibited EndoG samples were measured with DMSO only or DMSO with recEndoG (4 μg/mL), respectively. After addition of EndoG, fluorescence intensity was kinetically measured in a Bio-Tek Synergy 4.0 plate reader at 37°C. Mean velocity (mRFU/min) within 20 min was automatically calculated by the plate reader. Background was subtracted before the calculation of EndoG activity. The percentage of EndoG activity left after inhibition was calculated by the mean velocity of the test compound divided by the mean velocity of DMSO control with recombinant EndoG multiplied by 100. In similar assays, recombinant human DNase I (rhDNase I, Pulmozyme; Genentech, South San Francisco, CA) was used at a concentration of 60 ng/mL in 0.1 mM $CaCl_2$, 0.3 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4; 0.126 μg/mL DNase II (Worthington, Lakewood, NJ) was tested in 100 mM sodium citrate buffer, pH 5.0.

Plasmid incision assay

This method was used as a secondary assay for subsequent evaluation of initial hits. The detection was based on inhibition of EndoG-mediated plasmid DNA degradation assessed by gel electrophoresis. Activity was measured using a 20-μL sample containing 1 μg pECFP plasmid DNA, 0.1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 1 μL of test compound in DMSO, and recEndoG (mouse, 200 ng/mL, or human, 42 μg/mL). After a 1 h incubation at 37°C, the reaction was terminated by adding 2 μL of 10 mM Tris-HCl, pH 7.4, 1% SDS, 25 mM EDTA, and 7.2 mM bromophenol blue. The samples were run in 1% agarose gel as previously described (Buzder *et al.*, 2009). An EagleEye scanning densitometer (Stratagene, La Jolla, CA) was utilized to quantify the relative amount of endonuclease-treated plasmid DNA present in a covalently closed circular (supercoiled) DNA, open circular DNA, linear DNA, or digested form. One unit is defined as the amount of endonuclease that is capable of converting 1 μg of covalently closed supercoiled plasmid DNA to open circular, linear, or digested DNA in 1 h at 37°C.

Cell culture

Normal rat tubular epithelial NRK52E, human prostate carcinoma epithelial 22Rv1, and human prostate adenocarcinoma PC3 cells (all from ATCC, Manassas, VA) were grown, respectively, in Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, or F-12K medium, all of which were supplemented with 5–10% fetal bovine serum at 5% CO_2/95% air in a humidified atmosphere at 37°C, fed at 48–72 h intervals, and used within 1 day after confluence. Cisplatin (60 μM; APP Pharmaceuticals, Schaumburg, IL) and Docetaxel (80 μM; Sigma-Aldrich, St. Louis, MO) were then added to the cells for a 24 h incubation, followed by the measurement of cell death.

Cell death assay

The cytoprotective effects of the inhibitors were tested using lactate dehydrogenase (LDH) release assay (CytoTox96 Non-Radioactive Cytotoxicity assay kit; Promega, Madison, WI). In brief, 8000–10,000 cells per well in 96-well plates were incubated at 37°C for 24 h followed by a 2-h incubation in presence of an EndoG inhibitor.

EndoG overexpression

In some experiments, PC3 cells were transfected with pECFP-N1 vector encoding a human mature *EndoG* gene sequence. Transfection was performed with 0.2 or 0.7 μg plasmid DNA in 96-well cell culture plates (Costar) or an 8-well slide chamber (Lab-Tek, Rochester, NY), respectively, in the presence of Lipofectamine 2000 (Invitrogen, Carlsbad, CA), which was used according to the manufacturer's instructions.

Protein extraction

NRK52E cells were grown to ~80% confluence in 10-mm culture dishes. Medium was aspirated, and the cells were rinsed with ice-cold 1×PBS, pH 7.4. The cells were lysed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1% Triton X-100 for 10 min on ice and then sonicated for 15 s. Cell debris was removed by centrifugation at 13,000 *g* for 10 min at 4°C. The supernatant was collected and stored at −80°C until further use.

Other enzyme activities

LDH, protease, superoxide dismutase 1 (SOD1), and ribonuclease A (RNase A) activities in the cell extract were

measured by using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega), Protease Fluorescent Detection kit (Sigma-Aldrich), SOD determination kit (Sigma-Aldrich), and Ribonuclease A Detection kit (Sigma-Aldrich), respectively, according to the manufacturer's instructions.

TUNEL assay

PC3 cells fixed in 5% formalin were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using the *In Situ* Cell Death Detection Kit from Roche Diagnostics (Indianapolis, IN). Cells were rinsed, counterstained with 4,6-diamidino-2-phenylindole (DAPI) for DNA, and then mounted under cover slips with Prolong® Antifade kit (Invitrogen). Images were acquired using an Olympus IX-51 inverted microscope (Olympus America, Center Valley, PA) equipped with a Hamamatsu ORCA-ER monochrome camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan).

Image analysis

Image analysis was performed using SlideBook v.4.2 software (Olympus America). For quantification, 10 independent fields of view were collected for each subset of experiments, and mean optical density was recorded for the channels used. Blue and green colors were used to mask nuclei stained with DAPI and DNA fragmentation detected by TUNEL, respectively.

Statistics

Statistical analysis was performed using Student's *t*-test. Results were expressed as mean ± standard error of mean. $p < 0.05$ was considered statistically significant. Bonferroni modification of the Student's *t*-test was applied for *p* calculation where appropriate.

Results

Optimization of the HTS EndoG assay

For optimization of the HTS EndoG assay, reaction conditions, including cofactors, reaction time, enzyme, and the activity probe concentration, were tested. Recombinant EndoG was purified from *E. coli* and tested for activity homogeneity in zymogram gel as described in the Materials and Methods section. Recombinant EndoG consisted of a single band of 28 kDa (Fig. 1A), which corresponded to the mature enzyme. Varying concentrations of cofactors ($MgCl_2$, $MnCl_2$, or $CaCl_2$) were elaborated to reach the maximum activity. The EndoG activity measured as the

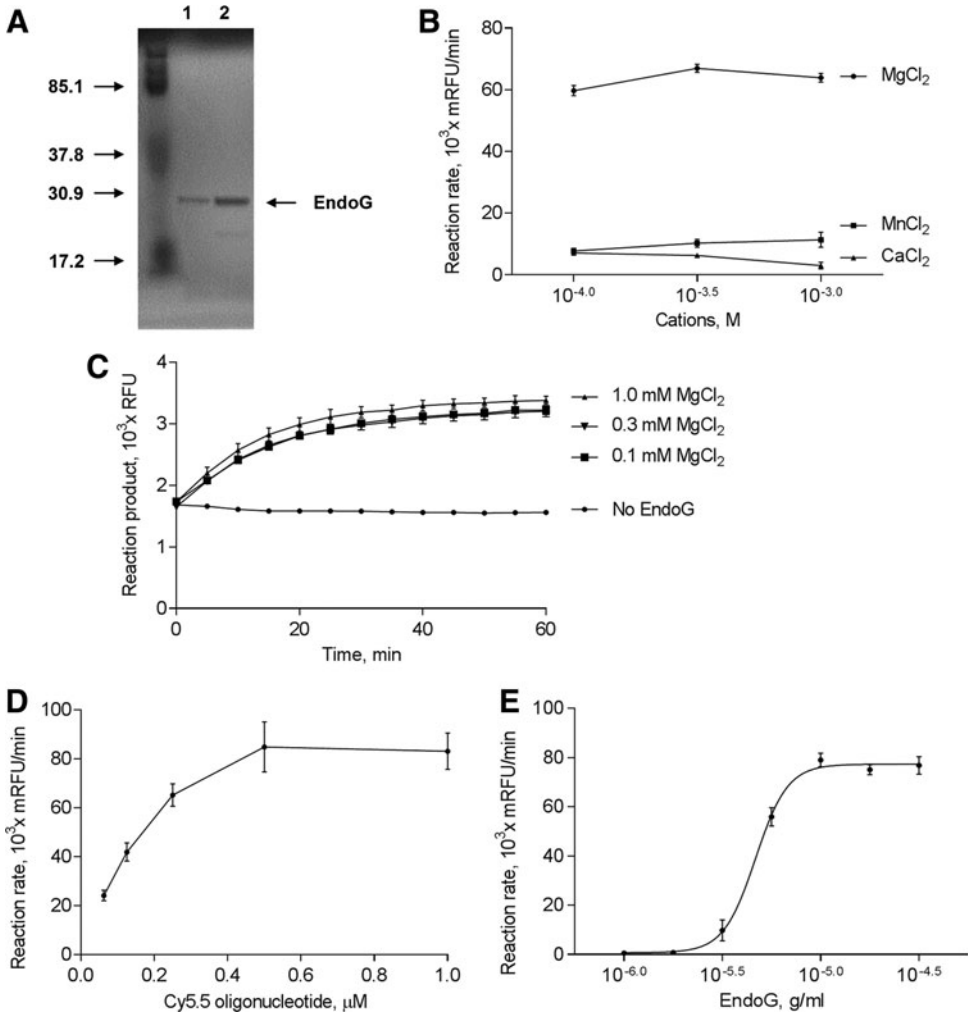

FIG. 1. Optimization of cofactors for the screening assay for endonuclease G (EndoG) inhibitors. **(A)** Zymogram gel electrophoresis of recombinant mouse EndoG. The 29 kDa endonuclease activity band is shown by the *arrow*. Lane 1, human EndoG (shown as control for comparison). Lane 2, mouse recombinant EndoG (recEndoG). **(B)** The effects of Ca^{2+}, Mg^{2+}, and Mn^{2+} ion concentrations. **(C)** Determination of the linear time range of the reaction. **(D)** The effects of varying concentrations of the Cy5.5-labeled oligonucleotide endonuclease activity probe. **(E)** A dose–response curve of EndoG to determine the enzyme's EC_{50} value (4.67 μg/mL).

reaction velocity (mRFU/min) was shown to maximize at 0.3 mM $MgCl_2$, while the effects of other cations were negligible (Fig. 1B). The reaction product increased in a time-dependent manner and close to linearity within the first 20–30 min of incubation at all of the tested concentrations of $MgCl_2$ (0.1–1.0 mM; Fig. 1C). Linear regressions (R^2) at each concentration between 0 and 20 min were performed to determine that 0.1 mM $MgCl_2$ had statistically ($p < 0.0001$) the best regression (0.817) among the concentrations. At this condition, varying concentrations of the probe were tested to determine the optimal concentration for screening. The EndoG activity (mean velocity) increased between the 0 and 1 μM probe, after which it seemed to level off (Fig. 1D). Therefore, the 0.25 μM probe concentration was chosen for further experimentation. The EC_{50} of EndoG under these optimized conditions was determined to be 4.67 μg/mL (Fig. 1E).

Determination of IC_{50} values of known EndoG inhibitors

Specific chemical inhibitors for EndoG in *in vivo* applications are not available. However, $ZnCl_2$, a universal inhibitor of all endonucleases, and EDTA, a chelator of bivalent cations and an inhibitor of all cation-dependent endonucleases, are useful for some *in vitro* applications. We tested $ZnCl_2$ and EDTA utilizing the optimized EndoG HTS assay mentioned earlier. The IC_{50}s of $ZnCl_2$ and EDTA were determined to be 0.94 μM (Fig. 2A) and 71.68 μM (Fig. 2B), respectively. Therefore, this optimized assay can be applied to HTS for new EndoG inhibitors.

Screening of an in-house chemical library for EndoG inhibitors

The HTS assay was applied to the screening of a chemical library containing 1040 chemical compounds at a concentration of 1 μM each. Pilot tests showed that EndoG activity was not compromised by 1% DMSO in this assay or cell culture assays described next (data not shown). In total, 17 candidate compounds were identified as inhibiting EndoG activity by more than 60% at a concentration of 1 μM (Fig. 3). These hits were processed for follow-up characterization.

Validation of hits from the screening assay by a secondary assay

To validate the inhibitory activities of the hits from the HTS assay, the PIA was used as a secondary test to confirm or reject candidate compounds. Out of the 17 hits obtained, two potent EndoG inhibitors, namely PNR-3-80 (5-((1-(2-naphthoyl)-5-chloro-1*H*-indol-3-yl)methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione) and PNR-3-82 (5-((1-(2-naphthoyl)-5-methoxy-1*H*-indol-3-yl)methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione), were identified because they protected the plasmid DNA from degradation by EndoG in the secondary assay (Fig. 4A). In presence of these two compounds (lanes 7 and 8) at 1 μM, most of the substrate, covalently closed super-coiled plasmid DNA, remained uncleaved compared with the negative control (lane 2); whereas the other 15 hits did not protect the substrate. The chemical structures of the two confirmed EndoG inhibitors are shown in Figure 4B and C. The two confirmed EndoG inhibitors were further tested by PIA against human recEndoG (Fig. 4D). PNR-3-30 (lane 4) and PNR-3-82 (lane 5) at 1 mM partially protected the substrate from degradation by human recEndoG compared with the negative control (lane 2).

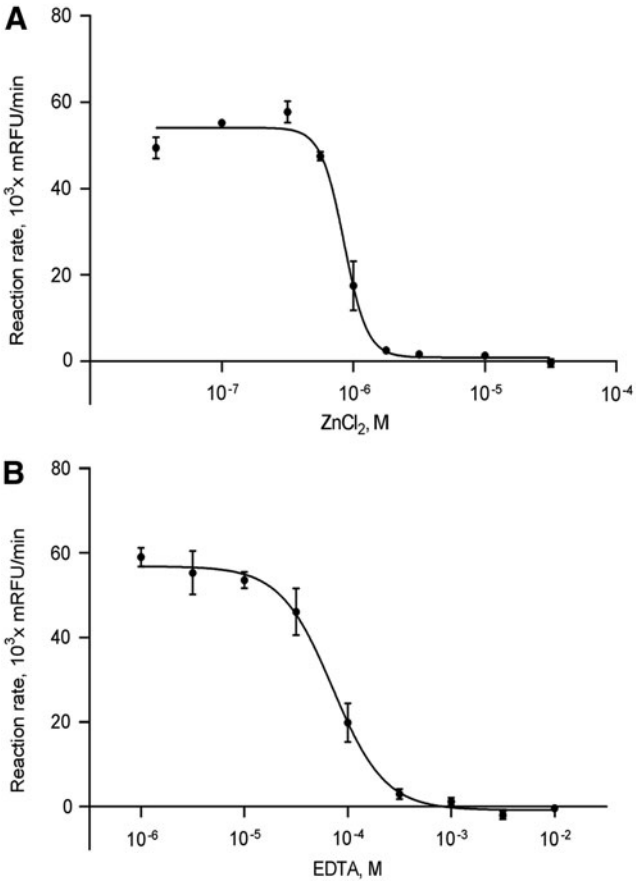

FIG. 2. Evaluation of EndoG inhibition by known *in vitro* inhibitors, $ZnCl_2$ and EDTA. IC_{50} values of **(A)** $ZnCl_2$ (0.94 μM) and **(B)** EDTA (71.68 μM).

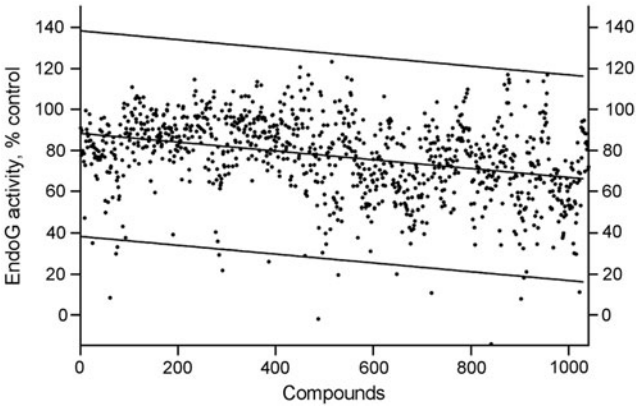

FIG. 3. Identification of EndoG inhibitor candidates after screening of an in-house chemical library. Screening results at 1 μM concentration produce 17 compounds hits showing more than 60% inhibition of EndoG activity. The *middle line* indicates the trend line.

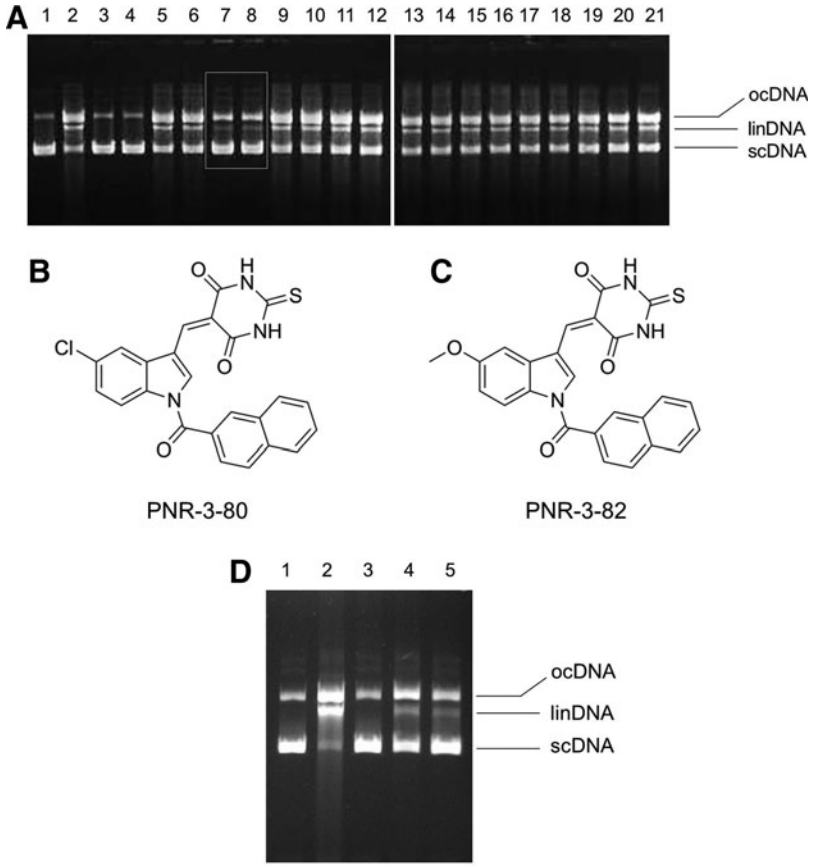

FIG. 4. Testing of potential EndoG inhibitors (hits) by secondary assay. **(A)** The plasmid incision assay (PIA) was performed as described in the Materials and Methods section. Lanes 1, 2, 3, and 4 indicate controls: 1% dimethylsulfoxide (DMSO) (no EndoG), EndoG with 1% DMSO, EndoG with $ZnCl_2$, and water (no EndoG), respectively. Potential EndoG inhibitors at 1 μM in 1% DMSO were tested in lanes 5–21. Two selected EndoG inhibitors (lanes 7 and 8) are indicated by the *white box*. DNA isoforms include open circular DNA, ocDNA; linear DNA, linDNA; and supercoiled DNA, scDNA. **(B, C)** Chemical structures of the two selected EndoG inhibitors, PNR-3-80 and PNR-3-82. **(D)** Two selected EndoG inhibitors were tested by PIA against human recEndoG. Lanes 1, 2, and 3 indicate controls: 1% DMSO (no EndoG), EndoG with 1% DMSO, and EndoG with $ZnCl_2$. The EndoG inhibitors, PNR-3-80 and PNR-3-82 (at 1 mM each), are shown in lanes 4 and 5, respectively.

Specificity of the inhibitors

Specificity of a potential inhibitor is important for any *in vitro* or *in vivo* application, and generally defines the potential usefulness of an inhibitor. In order to determine the specificity of the two candidate EndoG inhibitors, we first compared their IC_{50} values against EndoG with the IC_{50} values for two other endonucleases, namely DNase I and DNase II (Fig. 5A, B). These enzymes were chosen, because they represent the majority of endonuclease activity in most mammalian cells (Enari *et al.*, 1998; Apostolov *et al.*, 2009), and as they are commercially available as pure enzymes. Our data showed that PNR-3-80 and PNR-3-82 were ~17 and 104 times, respectively, more specific for EndoG than for DNase I. The two inhibitors showed almost no activity toward DNase II (Fig. 5C). Moreover, both of these EndoG inhibitors did not show any interference with the four other enzymes related to cell death, including RNase A, protease, LDH, and SOD1, at the two tested concentrations (0.1 and 10 μM; Fig. 6A–D). Based on our experience, we knew that ''normal'' kidney tubular epithelial NRK52E cells express high activities of the housekeeping enzymes, and, thus, the protein extract of these cells was a convenient model for these experiments. These data suggest that the identified inhibitors are highly specific to EndoG as compared with other tested enzymes that are both nucleases and non-nucleases.

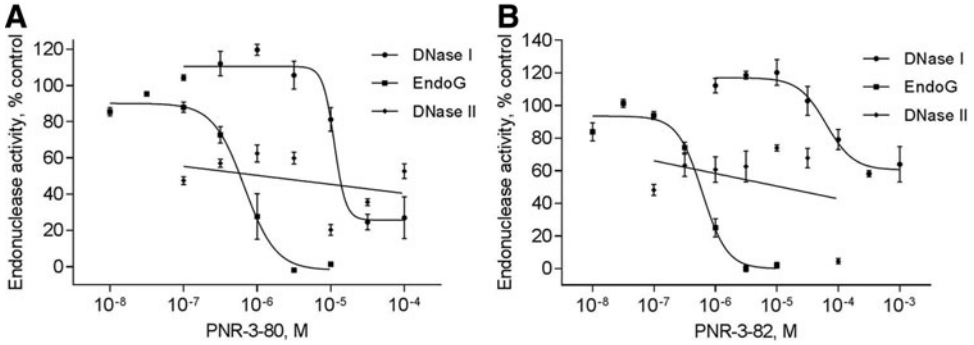

FIG. 5. Evaluation of the specificity of the two lead inhibitors to apoptotic endonucleases. **(A, B)** Determination of IC_{50}s. **(C)** Resulting IC_{50} values.

C

Inhibitors	PNR-3-80	PNR-3-82
EndoG	0.67 μM	0.61 μM
DNase I	11.21 μM	63.31 μM
DNase II	> 100 μM	> 100 μM

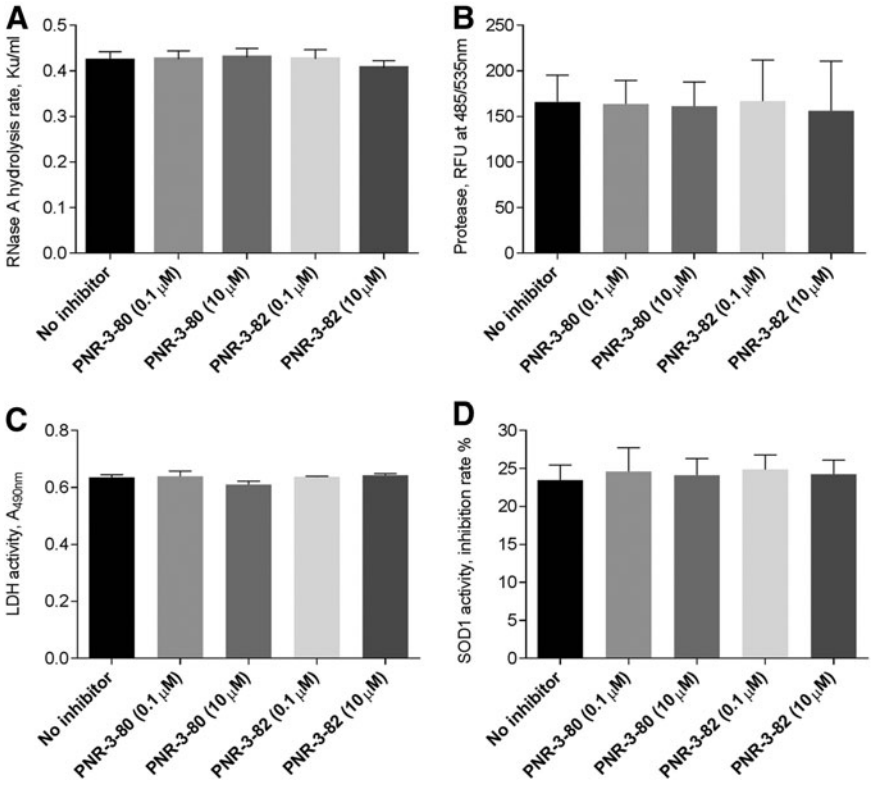

FIG. 6. Evaluation of the specificity of the two lead inhibitors for inhibition of other enzymes. Activities for **(A)** ribonuclease A (RNase A), **(B)** protease, **(C)** lactate dehydrogenase (LDH), and **(D)** superoxide dismutase 1 (SOD1) in NRK52E cell extracts were measured in presence of inhibitors at 0.1 and 10 μM.

Kinetic analysis of the EndoG inhibitors

To identify the mechanisms of the two EndoG inhibitors, the substrate was titrated with two concentrations (0.2 and 0.4 μM) of each inhibitor and Michaelis–Menten kinetics were plotted (Fig. 7A, C). The inhibitory patterns of the two inhibitors were examined using Lineweaver–Burk plots (Fig. 7B, D). According to the plots, they were identified as uncompetitive inhibitors (Cornish-Bowden, 1974), which bind only to the entire enzyme–substrate complex.

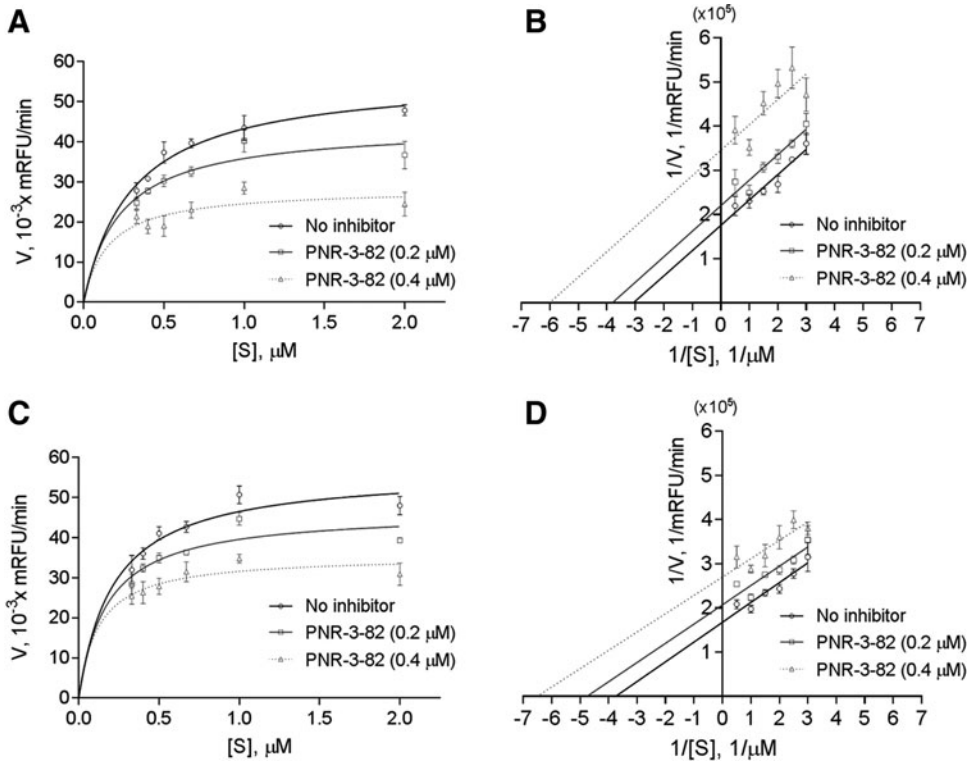

FIG. 7. Kinetic analysis of the two lead inhibitors. The inhibitory activities were determined at two concentrations each compared with the control without inhibitors. **(A, B)** Michaelis–Menten kinetics and Lineweaver–Burk plots for PNR-3-80. **(C, D)** Michaelis–Menten kinetics and Lineweaver–Burk plots for PNR-3-82. Based on the plots, the compounds are identified as uncompetitive inhibitors.

Cytoprotective activity of the EndoG inhibitors

It was previously shown that EndoG is necessary for cytotoxic activities of anticancer drugs in prostate cancer cells (Wang *et al*., 2008). Cells with low EndoG expression/activity are more resistant to anticancer drugs compared with the cells with high EndoG expression/activity. To test the cytoprotective activities of the two EndoG inhibitors, human prostate carcinoma epithelial 22Rv1 cells, which naturally express EndoG, were exposed to Cisplatin (60 μM) in presence or absence of each of the inhibitors. Cell death was then measured by the LDH release assay. In this experiment, PNR-3-80 showed significant inhibition of Cisplatin-induced cell death compared with the control (without inhibitor). PNR-3-82 also inhibited Cisplatin-induced cell death, but the effect was less than that of PNR-3-80 (Fig. 8A). Both inhibitors were then tested in a different cell model. In this model, we used human prostate cancer PC3 cells, because EndoG expression in this cell line is very low (Wang *et al*., 2008). As expected, the overexpression of EndoG in PC3 cells made them more sensitive to Docetaxel compared with untransfected cells, and both EndoG inhibitors significantly inhibited Docetaxel-induced cell death in these cells (Fig. 8B, C). Moreover, we observed that the two EndoG inhibitors significantly decreased DNA fragmentation in the TUNEL assay (Fig. 9A, B). Taken together, these data suggest that these EndoG inhibitors can protect cells from drug toxicity, likely by inhibition of EndoG.

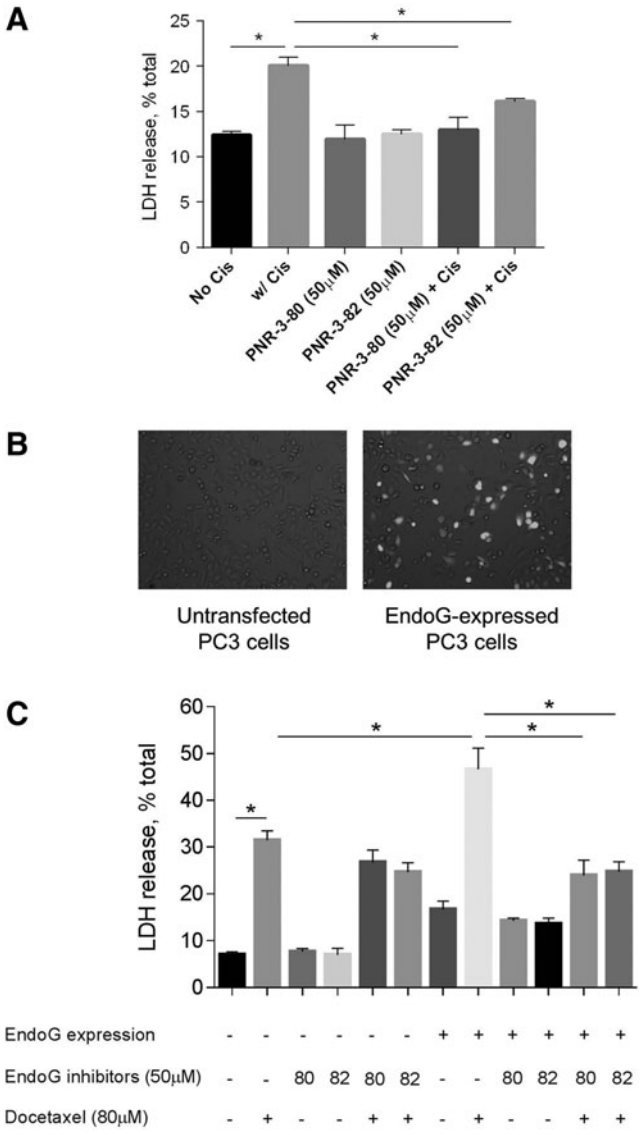

FIG. 8. Cytoprotective effect of the two lead inhibitors against anticancer drugs. **(A)** Inhibition of Cisplatin (60 μM)-induced cell death by inhibitors in cultured 22Rv1 cells using the LDH release assay. **(B)** Overexpression of EndoG in PC3 cells. **(C)** Cytoprotection by inhibitors, PNR-3-80 (80) and PNR-3-82 (82) (50 μM each), in EndoG-overexpressing PC3 cells against Docetaxel (80 μM) as measured using the LDH release assay. $*p > 0.05$.

Discussion

This study focused on identifying the first small-molecule inhibitors of a major apoptotic endonuclease, EndoG. To achieve this, we have developed a novel high-throughput assay for endonuclease inhibitor screening of an in-house chemical library. The assay utilizes a self-quenched fluorescent reporter DNA probe for the measurement of endonuclease activity. By applying recombinant EndoG as the reporter probe-degrading agent, we made the assay applicable for EndoG inhibitor screening. We then screened a

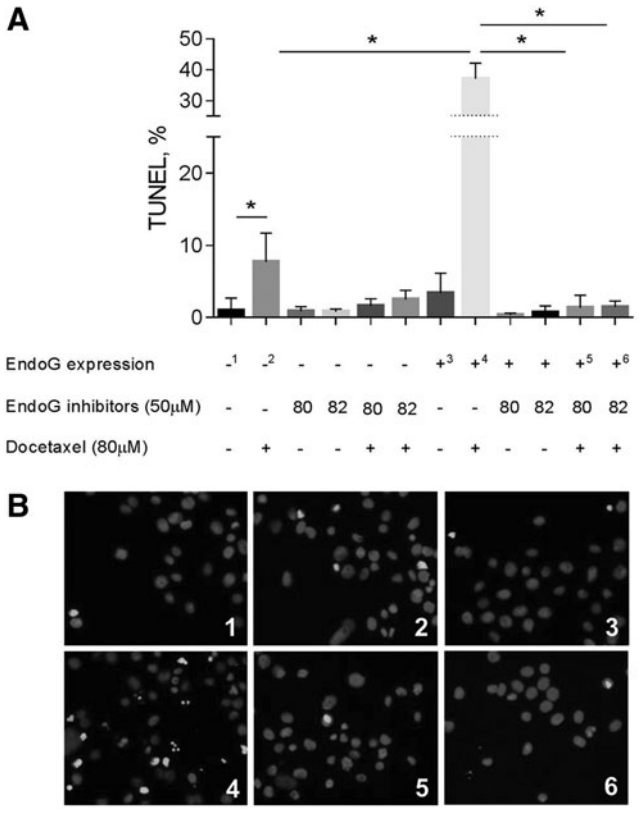

FIG. 9. TUNEL staining and quantification of EndoG-overexpressing PC3 cells after treatment with Docetaxel (80 μM) in the absence or presence of the two lead inhibitors, PNR-3-80 (80) and PNR-3-82 (82) (50 μM each). **(A)** TUNEL assay quantification. **(B)** Representative images. Pictures are shown in the order designated by *superscript numbers* in *x*-axis of **(A)**. $*p > 0.05$.

chemical library of 1040 compounds and identified two potent and selective EndoG inhibitors. The two resulting compounds, PNR-3-80 (5-((1-(2-naphthoyl)-5-chloro-1*H*-indol-3-yl) methylene)-2-thioxodihydropyrimidine-4,6-(1*H*,5*H*)-dione) and PNR-3-82, (5-((1-(2-naphthoyl)-5-methoxy-1H-indol-3-yl) methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione), are thiobarbiturate analogs, and are the most specific and potent inhibitors that EndoG has known so far. Their IC_{50}s for EndoG inhibition are 0.67 and 0.61 μM, respectively. This potency is higher than that of known inhibitors such as $ZnCl_2$ ($IC_{50} = 0.94$ μM) and EDTA ($IC_{50} = 71.68$ μM). As expected, PNR-3-80 and PNR-3-82 significantly protected EndoG-expressing cultured human prostate carcinoma cells, 22Rv1 and PC3, from Cisplatin- or Docetaxel-induced cell death. The purpose of this study was, certainly, not protecting cancer cells against anticancer drugs. The prostate cancer cells were used, because they are a convenient model as these cells express mainly EndoG among apoptotic endonucleases. Real application of these findings would be protection of normal cells, which have the same cell death mechanisms as cancer cells, against harmful effects of anticancer drugs. For example, the EndoG inhibitors may become useful for bioprotection against known adverse reactions of the studied drugs, such as nephrotoxicity of Cisplatin or neurotoxicity of Docetaxel (Loehrer and Einhorn, 1984; Milosavljevic *et al.*, 2010).

Before this finding, potential EndoG chemical inhibitors valuable for cell injury amelioration were not available. Zinc sulfate is known to inhibit endonuclease activity in solution and in cultured cells, but it is not specific to EndoG (Ueda and Shah, 1992). Moreover, a 40.6-kDa protein inhibitor of EndoG, EndoGI, has been found in *Drosophila melanogaster* (Temme *et al.*, 2009). This endogenous protein inhibitor is localized in the cell nucleus. It is specific to EndoG, but the therapeutic value of this protein inhibitor is negligible, because it is expressed only in *D. melanogaster* and as it is a protein, whose delivery to cells would be problematic. Inhibition of EndoG expression by siRNA for research purposes has been described (Basnakian *et al.*, 2006). Using siRNAs as pharmaceuticals is possible. However, it still remains an unchartered territory in drug development, as it requires gene delivery systems with all their potential adverse reactions.

This study contains the very first EndoG HTS assay designed to identify inhibitors of this enzyme. Currently, endonuclease activity is measured by the PIA (Basnakian *et al*., 2002), the ROPSA (Basnakian and James, 1996), the radial diffusion assay (Takeshita *et al*., 2000), or zymogram gel electrophoresis (Basnakian *et al*., 2005). The PIA and zymogram gel assays are performed through electrophoresis to detect DNA-degrading activity by endonucleases. These assays can measure endonucleases activity, albeit with a low accuracy. Endonuclease activity can also be detected by the radial diffusion assay, which is based on endonucleases diffusion in DNA-containing gel plates. In the ROPSA, the endonuclease activity can be quantified by the relative number of 3′-OH cleavages in ~24 h. In addition, these assays are not EndoG specific. Therefore, due to their disparate specificity and low precision, these assays are not suitable for HTS. On the other hand, the fluorescent-labeled oligonucleotide-based assay used in this study is rapid (~25 min), sensitive to EndoG, and precise for the *in vitro* measurement of EndoG activity in a 96-well plate format. Moreover, this assay has the potential for use on automatic robotic screening platforms based in 384-well or 1536-well plate formats after further refinement.

In the screening of the in-house chemical library, initial hits were 17 out of 1040 molecules screened (1.6% hit rate). The final concentration of the compounds, the sensitivity of the assay, the chosen library, and the criteria for hit selection might affect the initial hit rate. We used a relatively low concentration (1 μM) and a strict criteria for selection of candidates that exhibited a 60% or greater EndoG inhibition activity in the primary screen for minimization of false selections. The PIA that was used as a secondary assay was capable of confirming and identifying the best candidates from the selected hits. There is a concern that the solubility of the inhibitors, especially at higher concentrations, and the use of DMSO might present issues for subsequent *in vivo* and clinical applications. Therefore, an improvement of the solubility characteristics of the lead compounds through appropriate structural modification is under development.

Further investigation is necessary to understand the detailed mechanism of action of the EndoG inhibitors identified in this study. Subsequently, structural modification of the PNR series of inhibitors leading to higher potency, lower toxicity, and resistance will be explored through structure-activity strategies. In addition, investigation of other endonucleases utilizing the assay described earlier would prove useful in the discovery of novel DNase inhibitors.

In summary, we have developed an HTS EndoG assay for new EndoG inhibitors, which resulted in the discovery of two novel, highly specific, and potent EndoG inhibitors. These molecules can be used to protect cells and tissues from various injuries, including chemical/drug poisoning, hypoxia, or physical injury (Basnakian *et al*., 2006; Apostolov *et al*., 2007; Wang *et al*., 2008; Apostolov *et al.*, 2011).

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Disclosure Statement

No competing financial interests exist.

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