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A high-throughput screen for detection of compound-dependent phosphodiester bond cleavage at abasic sites

Marc C. Rideout [a, *], Benjamin Liet [a], Didier Gasparutto [b], Nathalie Berthet [a, **]

[a] Département de Chimie Moléculaire (DCM), Laboratoire Ingénierie et Interactions BioMoléculaires (I2BM), UMR-5250, ICMG FR-2607, CNRS, Université Grenoble Alpes (UGA), 570 Rue de la Chimie, BP-53, 38041 Grenoble Cedex 9, France
[b] Institut des Nanosciences & Cryogénie (INAC), SPrAM - UMR 5819 CEA/CNRS/Université Grenoble Alpes, Commissariat à l'Energie Atomique, 17 rue des Martyrs, F-38054 Grenoble Cedex 9, France

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ABSTRACT

We have employed a DNA molecular beacon with a real abasic site, namely a 2-deoxyribose, in a fluorescent high-throughput assay to identify artificial nucleases that cleave at abasic sites. We screened a 1280 compound chemical library and identified a compound that functions as an artificial nuclease. We validated a key structure-activity relationship necessary for abasic site cleavage using available analogs of the identified artificial nuclease. We also addressed the activity of the identified compound with dose titrations in the absence and presence of a source of non-specific DNA. Finally, we characterized the phosphodiester backbone cleavage at the abasic site using denaturing gel electrophoresis. This study provides a useful template for researchers seeking to rapidly identify new artificial nucleases.

1. Introduction

One of the most frequent forms of damage to the genome of an organism is the loss of DNA nucleobases [1,2]. These apurinic or apyrimidinic (AP) sites can arise spontaneously via the hydrolysis of the N-glycosidic bond or enzymatically during the initial steps of base excision repair (BER) carried out by DNA N-glycosylases [3]. The combined result of these activities is an estimated 10,000 AP sites generated per cell per day [1,4]. AP sites can have cytotoxic effects, blocking DNA replication and transcription, or mutagenic effects as a result of translesion synthesis by low-fidelity polymerases (reviewed in [5]). In addition, their presence in the genome can lead to enzyme-mediated strand breaks catalyzed by Topoisomerases acting near the AP site, further contributing to their cytotoxicity [6,7].

The cytotoxicity of AP sites is exploited in the treatment of certain types of cancer exemplified by the use of alkylating chemotherapeutics such as Temozolomide [8]. Temozolomide primarily methylates purines at several nucleophilic positions, namely the O6- and N7-positions of guanine and the N3-position of adenine [9,10]. Methylation results in a more labile N-glycosidic bond which significantly increases the rate of spontaneous depurination and therefore the number of potentially cytotoxic AP sites [2,11]. BER of generated AP sites appears to be responsible, at least in part, for acquired resistance to Temozolomide treatment [12–14]. Therefore, one strategy to improve the efficacy of Temozolomide, or potentially other alkylating chemotherapeutics, is to identify compounds that can disrupt the repair of AP sites. This strategy can have two distinct approaches: one, poison the enzymes that attempt to repair the lesion such as Apurinic/Apyrimidinic endonuclease-1 (APE1) [15] or two, exacerbate the damage at the AP site.

Promising enzymatic inhibitors of APE1 are relatively abundant thanks to recent searches employing high-throughput fluorescent molecular beacons [16–18]. In contrast, artificial nucleases that cause phosphodiester backbone cleavage at AP sites have traditionally been identified using low-throughput plasmid-based assays (e.g., the tripeptide Lys-Trp-Lys [19] or aminoglycoside antibiotics [20] or rationally-designed hybrid molecules consisting

Abbreviations: AP, Apurinic/Apyrimidinic Site; APE1, Apurinic/Apyrimidinic Endonuclease 1; BER, Base Excision Repair; Fpg, Formamidopyrimidine DNA glycosylase; FITC, Fluorescein Isothiocyanate; nsDNA, non-specific DNA; RFU, Relative Fluorescence Units; MX, methoxyamine.

* Corresponding author.
** Corresponding author.
E-mail addresses: rideout.marc@gmail.com (M.C. Rideout), nathalie.berthet@ujf-grenoble.fr (N. Berthet).

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of a DNA intercalator tethered to a nucleobase via a polyamine chain [21–24]). In order to facilitate rapid screening of compound libraries for the identification of new artificial nucleases we employed a molecular beacon capable of monitoring compound-dependent AP site cleavage in real-time. The beacon is a DNA hairpin consisting of a 2-deoxyribose AP site, a fluorescent donor and a collisional acceptor at the 5′ and 3′ ends, respectively (Fig. 1A). In contrast to similar beacons that employ an AP site analog (i.e., THF) [25], the 2-deoxyribose sensitizes the beacon to compounds that cause β- (or β-/δ-) elimination reactions at the AP site (Fig. 1A; see Ref. [26] for review of mechanism). Cleavage of the phosphodiester backbone at the AP site liberates a 3′ oligonucleotide fragment that spontaneously disassociates, carrying with it the quencher, and the result is a real-time fluorescent output.

Herein, we describe the use of this molecular beacon in a high-throughput assay format to identify artificial nucleases. To validate our assay we screened a 1280-compound library and identified a compound that causes cleavage at the AP site. Using analogs of the identified compound we validated a key structure-activity relationship necessary for the AP site cleavage. Finally, the nature of phosphodiester backbone cleavage was characterized by denaturing gel electrophoresis using control enzymes that catalyze distinct cleavage reactions at AP sites, namely APE1 and Formamidopyrimidine DNA glycosylase (Fpg, Fig. 1A). Use of the 2-deoxyribose molecular beacon in this fashion will prove useful for researchers seeking to identify new artificial nucleases.

2. Materials and methods

2.1. Molecular beacon synthesis and purification

In order to prepare a large amount of material for high-throughput screening, we synthesized the molecular beacons on an ABI 3400 DNA synthesizer using standard phosphoramidite chemistry and commercially available reagents. Specialized reagents were purchased from Glen Research (West Virginia, USA), namely Dabcyl support resin (Catalog # 20-5912-41), 5′-Fluorescein Isothiocyanate (FITC) phosphoramidite (Catalog # 10-5901-90), and either Abasic II phosphoramidite for the AP beacon (Catalog # 10-1927-90), or dSpacer (Catalog # 10-1914-90), for the THF beacon. Both the AP and THF beacons have the same DNA sequence: 5′-FITC d (TCA CCT CGT GAC TTT TTT TTA GTC ACG (X) GGT GA) dabcyl-3′, where “X” represents the abasic site and is either 2-deoxyribose (AP) or THF. Coupling conditions and deprotection were according to the manufacturer's instructions, followed by ethanol/sodium acetate precipitation. Oligonucleotides were resuspended in nanopure water and purified via HPLC over a Nucleosil C18 column (Macherey-Nagel) using 50 mM triethylammonium acetate (A) and acetonitrile (B) as a buffer system. Oligonucleotides were eluted using a linear gradient from 5 to 35% Buffer B over 30 min while monitoring both the DNA (258 nm) and FITC/Dabcyl (495 nm) absorbance wavelengths. Pure fractions were desalted by passage over an illustra NAP-25 column (GE, Cat # 17-0852-01), eluted with water and then stored at −20 °C. DNA concentrations were quantified using calculated extinction coefficients.

2.2. Library screening and compound characterization

A detailed description of the 1280-compound library can be found in the supplemental information. All fluorescence assays were performed in 96-well black, flat-bottom, half-area plates coated with a non-binding surface (Corning # 3686), and all reactions were monitored in a PolarStar Omega fluorimeter at 37°C (Excitation (Ex) 485 nm, Emission (Em) 520 nm). The library was

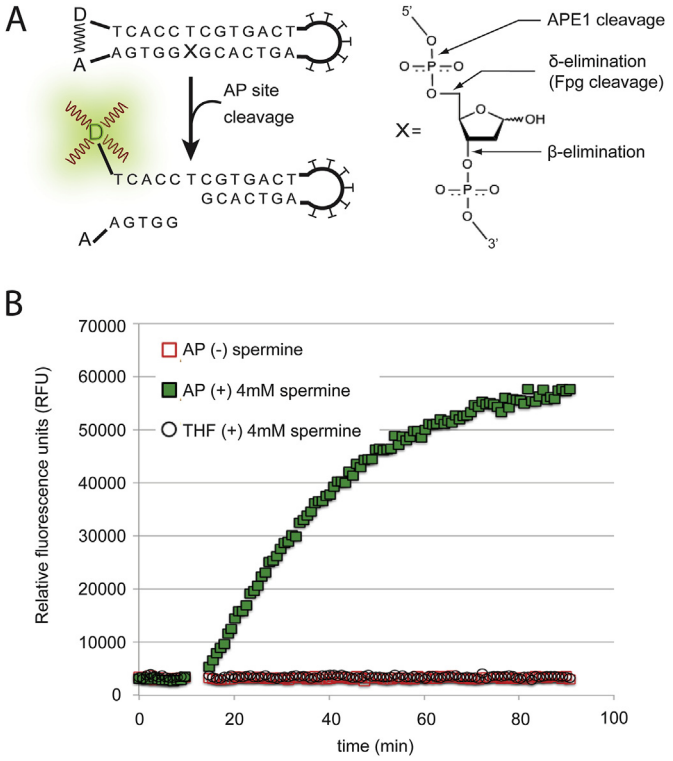

Fig. 1. DNA molecular beacon capable of detecting compound-dependent phosphodiester backbone cleavage at an abasic (AP) site in real-time. (A) Shown is the exact sequence of the DNA hairpin with a seven-thymidine loop. When the beacon is folded the fluorescent donor (D = 5′ FITC) and the collisional quencher (A = 3′ Dabcyl) are in close proximity and fluorescence is quenched. When cut at X (2-deoxyribose AP site) the acceptor disassociates and the beacon fluoresces. Arrows indicate bonds that are cleaved by the activity of Apurinic/Apyrimidinic endonuclease-1 (APE1), bifunctional DNA N-glycosylases such as Formamidopyrimidine DNA glycosylase (Fpg), or by compounds capable of causing β- (or β-/δ-) elimination reactions. (B) Inclusion of 4 mM spermine causes a real-time fluorescent readout following cleavage of the beacon containing the 2-deoxyribose AP site. Spermine is incapable of cleaving the analogous THF-containing beacon. Both the AP and THF beacons perform equivalently in cleavage assays with APE1 (Supplementary Fig. 1).

initially screened in 20 μL reactions at a final compound concentration of 1.25 mg/mL, 12.5% DMSO and 1.25 × NEBuffer 4 from New England BioLabs (NEB; 1× NEBuffer 4 = 50 mM Potassium Acetate, 20 mM Tris-Acetate, 10 mM Magnesium Acetate, 1 mM DTT, pH 7.9). For the initial screening, each compound was given 10 min to incubate in the plate while fluorescence readings were measured. Reaction rates (RFU/sec) for the initial screening were calculated for all data points, post-compound addition, that had a correlation coefficient (R^2) ≥ 0.9 between RFU and time. The median rate of these data points is used to describe the reaction velocity (Supplementary Table 1).

Following the initial screening, compound B8D7 and its structural analogs were sourced from powder stocks maintained by the Synthèse et Réactivité en Chimie Organique (SERCO) group at the Université Grenoble Alpes; NMR spectra for B8D7 and its structural analogs can be found in Supplementary Figures 2–4. Compound resuspensions and dilutions were performed in 100% DMSO. Non-specific DNA from salmon testes (Sigma Catalog # D-1626) was sheered by sonication to an average size of 500 base pairs (b.p.; verified by electrophoresis). Unless otherwise indicated, order of addition for all remaining reactions was water, NEBuffer 4 (1× final), 0.5 pmol of molecular beacon, then 0.63 pmol (200 ng) of nsDNA (if indicated), followed by either test compounds (2.5 μL), spermine or solvent controls, bringing the final reaction volume to 25 μL. Reactions with B8D7 and its structural analogs were