

Diastereomeric Recognition of 5',8-cyclo-2'-deoxyadenosine Lesions by Human poly(ADP-ribose) Polymerase 1 in Biomimetic Model

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Abstract 5',8-Cyclo-2'-deoxyadenosine (cdA), in the 5'*R* and 5'*S* diastereomeric forms, are typical non strand-break oxidative DNA lesions, induced by hydroxyl radicals, with emerging importance as a molecular marker. These lesions are exclusively repaired by nucleotide excision repair (NER) mechanism with a low efficiency, thus readily accumulating in the genome. Poly(ADP-ribose) polymerase1 (PARP1) acts as an early responder to DNA damage and plays a key role as a nick sensor in the maintenance of the integrity of the genome by recognizing nicked DNA. So far, it was unknown whether the diastereomeric cdA lesions could induce specific PARP1 binding. Here we provide the first evidence of PARP1 to selectively recognize the diastereomeric lesions 5'*S*-cdA and 5'*R*-cdA *in vitro* as compared to deoxyadenosine in model DNA substrates (23-mers) by using circular dichroism, fluorescence spectroscopy, immunoblotting analysis and gel mobility shift assay. Several features of the recognition of the damaged and undamaged oligonucleotides by PARP1 were characterized. Remarkably, PARP1 efficiently binds to both cdA lesions in the double stranded (ds)-oligonucleotides. In particular, PARP1 proved to bind 5'*S*-cdA with a higher affinity constant for the

5'S lesion in a model of ds DNA than 5'R-cdA, showing different recognition patterns, also compared with undamaged dA. This new finding highlights the ability of PARP1 to recognize and differentiate the distorted DNA backbone in a biomimetic system caused by different diastereomeric forms of a cdA lesion.

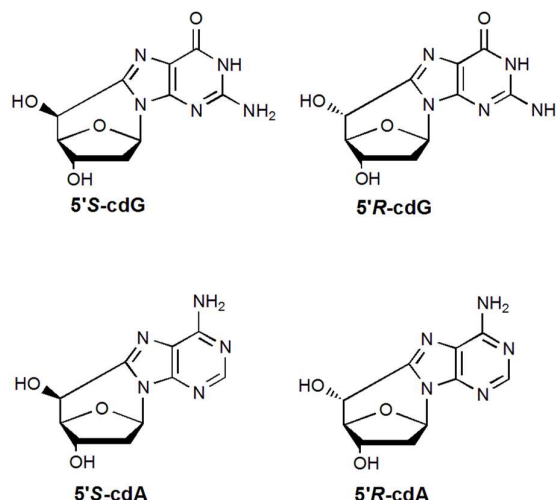
Keywords: human poly(ADP-ribose) polymerase 1 (PARP1), PARP-DNA complex, DNA-protein binding, DNA repair, 5',8-Cyclopurine-2'-deoxynucleoside, DNA damage, DNA repair efficiency.

1. Introduction

Purine 5',8-cyclo-2'-deoxynucleosides (cdPu), i.e., 5',8-cyclo-2'-deoxyadenosine (cdA) and 5',8-cyclo-2'-deoxyguanosine (cdG), are typical oxidized DNA lesions induced by an hydroxyl radical (HO•) abstraction of the H5' atom of the 2-deoxyribose moiety, followed by radical cyclization and oxidation steps, which creates a new covalent bond, locking the sugar and purine moieties [1,2]. These lesions can exist in the two diastereomeric forms 5'R and 5'S (Figure 1a) [1,2], are highly mutagenic *in vitro* and *in vivo* [3,4] and their accumulation in cellular DNA is associated with aging, cancer and neurodegeneration [5-9].

Numerous studies have shown that the concentrations of O₂, higher than physiological conditions, can inhibit the formation of cdPu lesions by reacting with the C5' radical, thereby preventing the cyclization reaction [10-12]. As the detection and quantification of these lesions are concerned, they don't suffer from oxidative artifacts during work-up, like the well known 8-oxo-dG [2,12]. The significant resistant of the glycosidic bond of cdPu to hydrolysis along with the proven oxygen stability make these lesions as robust biomarker of oxidative DNA damage, in particular caused by hydroxyl radical.

a) Diastereomeric purine 5',8-cyclo-2'-deoxynucleosides:



b) Experimental sequences:

5'-d(GCA GAC ATA TCC TAG AGX CAT AT)-3'

ss-N for X=dA

ss-5'S for X=5'S-cdA

ss-5'R for X=5'R-cdA

Figure 1. Chemical structures and oligonucleotide sequences. (a) Chemical structures of the diastereomeric purine 5',8-cyclo-2'-deoxynucleosides. (b) The sequences of the single stranded (ss) oligonucleotides used in this study. In the double stranded (ds) oligonucleotides, the template strand contains a ds-N (dA), or ds-5'S or ds-5'R and the complementary strand contains a T opposite to X.

In the past decade, increasing numbers of chemical and biological studies on cdPu lesions have been performed. A recent review summarized the latest results that demonstrate the implications of cdPu in several areas including DNA repair, biological effects, structural information and the association with human diseases [13].

The repair of cdPu lesions, in both diastereomeric forms, has been thoroughly studied and attributed to the nucleotide excision repair (NER) with different efficiency [13-15], i.e. the 5'R isomer being two times more efficiently repaired by NER than the 5'S isomer [15]. Molecular modeling and dynamics simulation elucidated that the different efficiency of NER is associated with the greater DNA backbone distortion caused by the 5'R isomer compared to the 5'S diastereomer [15,16] of the lesion. It has been found that these lesions are removed with a low efficiency by NER compared to other bulky DNA adducts [14], thereby leading to the accumulation of these oxidative lesions in the genome [1,14]. Consequently this can result in the stalling of DNA polymerases at a DNA replication fork [17-19], further leading to an efficient bypass of a 5'R-cdA, but inefficient bypass of a 5'S-cdA by DNA polymerase β (pol β) during DNA replication and base excision repair (BER) [20,21]. This

indicates that the DNA backbone distortions induced by the two diastereomeric cdPu lesions play a crucial role in modulating DNA repair efficiency.

In addition, the results from a competitive transcription and DNA adduct bypass assay have shown that 5'S-cdG also strongly inhibits transcription *in vitro* as well as in mammalian cells, thereby inducing transcriptional mutagenesis both *in vitro* and *in vivo* [4].

Moreover, the crystal structures of human polymerase η (Pol η) bypassing 5'S-cdA in insertion was reported in low resolution, indicating that DNA Pol η is most efficient in nucleotide incorporation opposite a cdA and least adequate in extending the primer promptly following the modified nucleoside [22]. Another study has also shown elevated steady-state levels of cdPu lesions in mice lacking endonuclease VIII-like 1 (NEIL1), a DNA glycosylase that removes an oxidized DNA base lesion and initiates BER; this enzyme is unable to directly repair these lesions while it can cooperate with the NER pathway in repairing the lesion [23]. Furthermore, it was reported that in *C.elegans*, endonuclease III homolog (NTH-1), can bind to cdPu lesions indicating that BER enzymes can bind to the lesions, but fail to remove them [13,24].

These findings have also shed light on the peculiar recognition of the 5'S in comparison with the 5'R lesion regarding the NER or pol β efficiency and on the induction of transcriptional mutagenesis. Moreover, it has been shown the first evidence for the unique and wide ranging effects of cdPu lesions on DNA helicases that are highly likely to encounter the abundant and stable oxidized base damage [25]. These results prompted us to investigate on the possibility that other DNA repair proteins may play a cooperative or facilitator role in the selective diastereomeric recognition. We focused on human poly(ADP-ribose) polymerase 1 (PARP1), in the recognition of 5',8-cyclo-2'-deoxyadenosine (cdA), in both 5'R and 5'S diastereomeric forms, which significantly distorts the DNA backbone.

Indeed, PARP1, belonging to the seventeen-membered superfamily of PARPs, is an enzyme that is activated by DNA damage responses cascade and a regulator of DNA repair including BER, homologous recombination, non-homologous end joining pathways as well as NER [26-28]. PARP1 can bind to single or double strand break intermediates [29,30]. It also plays an important role in gene transcription and chromatin remodelling in responding to distorted undamaged and damaged DNA structures, protecting the integrity of the genome and facilitating DNA repair. It has been indicated that PARP1 functions in the initial steps of damage recognition in global genome nucleotide excision repair (GG-NER), which is a dominant sub pathway of NER.

Especially, PARP1 binds to the DNA damage-binding protein 2 (DDB2) and its interaction with DDB2 at chromatin impairment by UV radiation stimulates its catalytic efficiency [31,32]. It is postulated that PARP1 can recognize DNA lesions that can distort the DNA backbone such as a thymine dimer induced by UV irradiation [33], but no data are available on the other lesions, such as the four diastereomeric cdPu lesions that can distort the DNA backbone. Recent studies, based on the SIRT1 inhibition by the activation of PARP-1 and the strongly associated NAD⁺ metabolism with the DNA damage responses through PARP1 [34,35], indicated that mitochondrial and mitophagic dysfunction through PARP-1 hyperactivation and NAD⁺/SIRT1 reduction may be implicated in XP-A patients neurodegeneration, revealing a progressive aging phenotype [36]. Furthermore, it has been indicated that CSB is as substrate for poly(ADPribose)ylation by PARP-1 after oxidative stress, implicating a functional interaction between PARP-1 and the role of CSB in the response to oxidative damage [37], while stalled transcription at DNA secondary structures located in ribosomal DNA are associated with PARP-1 activation in Cockayne syndrome and loss of CSA or CSB assembles on

mitochondrial dysfunction [38]. In this study, we provide the first evidence by characterizing PARP1 binding to the 5'R and 5'S isomers of cdA lesions. We examined PARP1 binding to double stranded (ds) oligonucleotide (23 bases) DNA substrates containing 5'R- and 5'S-cdA diastereomers located at the sixth nucleotide from the 3'-end (Figure 1b). The affinity of binding between PARP1 and the substrates with the lesions were examined by distinct approaches such as circular dichroism (CD), fluorescence spectroscopy, immunoblotting analysis and gel mobility shift assay. We anticipate that PARP1 can interact both with the undamaged and the damaged substrates with incorporated cdA lesions in *R* and *S* diastereomeric form, exhibiting simultaneously different affinity in binding to the modified and unmodified sequences. These evidences can obtain new insights into the recognition and discrimination features of cdPu diastereomeric lesions by DNA repair machinery.

2. Materials and Methods

The radionuclides [γ -³²P] ATP (6000 mCi/mmol) and Cordycepin in 5'-triphosphate 3'-[γ -³²P] (5000 mCi/mmol) were purchased from PerkinElmer Inc. (Boston, MA, USA). Human PARP1 (AG-40T-0011) and mouse PARP2 (AG-40T-0012) were purchased from AdipoGen (Liestal, Switzerland). Both enzymes were purified by affinity chromatography (purity: $\geq 99\%$ and $\geq 98\%$, respectively) and provided with a specific activity ≥ 600 U/mg protein.

All other chemical reagents and solvents were purchased from Link Technologies, Sigma Aldrich, Fluka, and Carlo Erba and used as received. Deionized distilled water (Mill-Q) was used for HPLC and RP chromatography.

2.1. General methods for synthesis and purification of oligonucleotides (ODNs)

ODNs were prepared by automated synthesis using the DMT- and β -(cyanoethyl) phosphoramidite method, on CPG supports (500 Å), with an Expedite 8900 DNA synthesizer (*Applied Biosystems*) at the 1 μ mol scale. Following to their synthesis, the DMTr-on ODNs were cleaved from the solid support and deprotected by the method of two syringes using AMA reagent [NH_4OH (30%)/ CH_3NH_2 (40%) 1:1] for 10' at room temperature. The AMA solution containing the cleaved ODN was placed in a sealed vial and heated for 15' at 55°C. The solvent was then removed in a Speedvac.

The crude 5'-DMT-on oligomers were purified and detritylated on-column by RP-HPLC (Grace Vydac C18 column, 5 μ m, 50x22 mm).

The ODNs were further purified by SAX HPLC (preparative DNA Pac PA-100 column, 5 μ m, 22x250 mm). TRIS-HCl 25 mM, pH=8 (buffer A) and TRIS-HCl 25mM, NaClO₄ 0.5M, pH 8.0 (buffer B) were used at a flow rate of 9 mL/min eluting with 2-30 % B in 30 min, 30% B for 10 min, then 30-45% B in 5 min monitoring at 254 nm. The purified fractions were concentrated, desalted on *Water SepPak*TM-C₁₈-cartridges and lyophilized again.

The final DNA yield was estimated by UV absorption in aqueous solution measured at 254 nm on a Cary 100 UV/Vis Spectrometer following standard procedures. MALDI-TOF mass spectrometry and analytical SAX HPLC chromatography were used to characterize the purified ODNs.

2.2. Preparation of double stranded oligonucleotide substrates

The oligonucleotide strands 5'-d(GCA GAC ATA TCC TAG AGA CAT AT)-3' (ss-N), or 5'-d(GCA GAC ATA TCC TAG AGX CAT AT)-3' where X is 5'S-cdA for ss-5'S and 5'R-cdA for ss-5'R,

were annealed to the complementary strands in equimolar concentrations in buffer solution containing 10 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, pH 7.2. The substrates were constructed by heating the two strands of the substrates at 90°C for 10 min and subsequently allowing the temperature to slowly drop down to the room temperature (25 °C).

Melting temperatures (T_m) of the substrates were measured with a Cary 100 UV/Vis spectrometer using a 1 mL quartz cuvette with a 1 cm pathlength. This allowed to monitor the absorbance of the solutions at 260 nm as a function of temperature. The temperature cycles were recorded from 20 to 80 °C per each strand with a temperature controller at a heating rate of 0.3 °C/min (Table 2, Figure 2).

2.3. Circular dichroism

CD spectra were recorded on a *Jasco J-710* spectropolarimeter using a quartz cuvette (0.1 cm optical path length) at a scanning speed of 50 nm/min with 1 s response time. Measurements at the range of 200–360 nm were the average of four accumulations at 295 K and smoothed with Origin, Version 8.00 program. CD profiles of the binding of PARP1 and PARP2 proteins (15 $\mu\text{g}\cdot\text{mL}^{-1}$) to the increasing concentrations of double stranded oligonucleotide substrates with a dA or diastereomeric cdA lesions (50 nM, 100 nM and 200 nM) were obtained in phosphate buffered saline (PBS buffer, 10 mM NaH_2PO_4 , 100 mM NaCl, 0.1 mM EDTA, pH 7.2). The reported spectra are differential spectra, i.e. they were obtained by subtracting the spectrum of each oligo substrate from that obtained from the corresponding PARP-substrate complex. The secondary structures of PARP proteins were determined by the far-UV CD spectra using the CONTIN software package [39]. Each CD analysis was repeated three times. Ellipticity values were converted into molar ellipticity, Θ ($\text{deg}\cdot\text{cm}^2\cdot\text{nmol}^{-1}$) based on the molecular weight of PARP1 proteins.

2.4. Fluorescence spectroscopy

Fluorescence spectra were recorded with an Edinburgh FLS 920 Fluorimeter (continuous 450 W Xe lamp for excitation), equipped with a Peltier-cooled Hamamatsu R928 photo multiplier tube for detection in right angle mode. Steady state fluorescence spectra of air-equilibrated solutions in the presence of PARP protein alone or the presence of both PARP proteins and the substrates in PBS buffer under the same experimental conditions as those for measuring CD spectrum. The fluorescence was measured with selective excitation at 295 nm (excitation wavelength of tryptophan), in a small square cell (5x5 mm) [39]. All experiments were conducted in triplicate.

2.5. Immunoblotting Analyses

Polyacrylamide (8%) and immunoblotting were performed according to the method from Faraone-Mennella *et al* [40]. The binding mixtures (9 μL) of PARP1 (0.15 μg), in the presence and absence of oligonucleotides were assembled in PBS buffer and diluted to 12 μL with sample buffer (60 mM Tris-HCl, pH 6.8, 1% SDS, 140 mM 2-mercaptoethanol, 20 % glycerol, 0.15 % bromophenol blue). PARP1-oligonucleotide complexes were stable under electrophoresis conditions, even in the presence of detergent and reducing agent, as described in [33,40,41]. Sample boiling was avoided.

Before loading, samples were incubated at room temperature in sample buffer, containing bromophenol blue, for 10 minutes. Gel electrophoresis was performed at 130 volt 25 mA for 40 minutes. PARP proteins were then electro transferred onto a PVDF membrane (0.2 μm Bio Rad) at

200 mA for 2 hours at 4°C in the electrophoresis buffer with 0.025% SDS. A polyclonal anti-PARP1 primary antibody in rabbit (H-250, Santa Cruz, 1:1000, v/v) was used to detect PARP1 proteins. The epitope corresponded to aminoacid 764-1014 at C-terminus of human PARP-1 (catalytic site). The antibodies which bound to PARP1 proteins were detected with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (IgG) from Bio-Rad Laboratory (Milan, Italy), which were subsequently incubated with the substrates producing HRP chemiluminescence (Super Signal West Dura Extended Substrate, 34075, PIERCE) that was measured by Chemidoc XRS (BioRad, Milan, Italy).

2.6. Gel mobility shift assay

A double stranded DNA substrate with a dA or diastereomeric cdA lesions opposite T in the template strand was employed to measure the binding of PARP1 to the DNA bases. A substrate was created by annealing a DNA strand with a dA or cdA to a complementary strand at ratio of 1:2. Substrates were radiolabeled at the 5'-end of the strand containing a dA or cdA. Substrates (10 nM) were incubated with various concentrations of PARP1 (30, 40 and 50 nM) in binding buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mg/mL bovine serum albumin, 0.1% Nonidet P-40 and 5 % glycerol on ice for 8 min. PARP1-oligonucleotide complex was then separated from free DNA by agarose (1%)-acrylamide (0.1%) gel electrophoresis and detected by a Pharos FX Plus Phosphor Imager from Bio-Rad (Hercules, CA).

3. Results and Discussion

3.1. Synthesis and characterization of 23-mer oligonucleotides containing dA and cdA lesions

The phosphoramidites of the two 5'R and 5'S-cdA nucleosides were prepared following the radical-based protocols developed previously [42]. The modified and unmodified 23-mer oligo-2'-deoxyribonucleotide (ODN) sequence 5'-d(GCA GAC ATA TCC TAG AGX CAT AT) with X=5'R- or 5'S-cdA or dA were synthesized by automated synthesis and purified following the procedures described previously [43]. More specifically, after standard deprotection with AMA reagent [NH₄OH (30%)/CH₃NH₂ (40%)] the crude 50-DMTr-on ODNs were detritylated and purified by reversed phase HPLC. Further purification was carried out by strong anion-exchange (SAX) HPLC and the purity and homogeneity of the collected fractions was monitored by analytical strong anion-exchange (SAX) HPLC (Figure S1). The molecular weights of the ODNs were assessed by MALDI-TOF in the negative mode (Table 1, Figure S2).

Table 1. Sequences and molecular masses of the synthesized ODNs

Strands	Sequence (5'-3') ¹	Mass calcd (Da)	Mass found ² (Da)
ss-N	GCA GAC ATA TCC TAG AGA CAT AT	7040.7	7038.1
ss-5'S	GCA GAC ATA TCC TAG AGX CAT AT	7038.7	7037.2
ss-5'R	GCA GAC ATA TCC TAG AGX CAT AT	7038.7	7036.9
CS ³	ATA TGT CTC TAG GAT ATG TCT GC	7044.7	7044.7

¹ X is 5'S-cdA for ss-5'S and 5'R-cdA for ss-5'R.

² All the oligonucleotide masses were obtained by MALDI-TOF in negative mode. [M – nH][–]

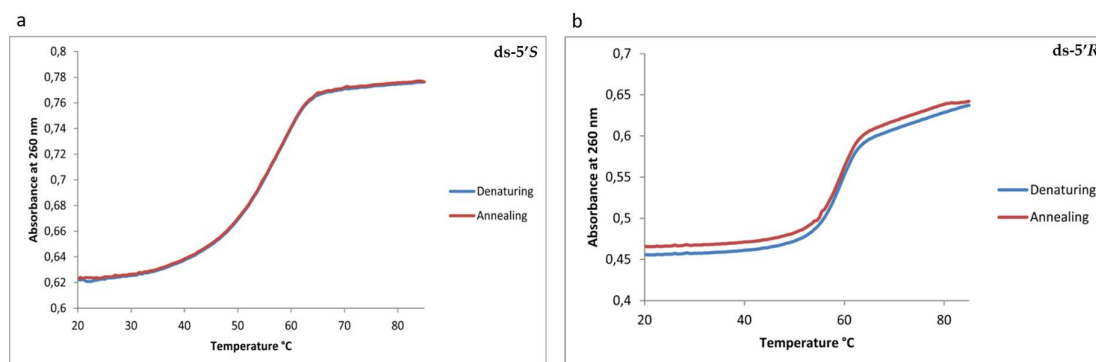
³ CS = Complementary strand

3.2. Thermal destabilization of modified oligonucleotide duplexes by diastereomeric cdA lesions

The thermal stabilities of the 23-mer duplexes 5'-d(GCA GAC ATA TCC TAG AGX CAT AT) • 3'-d(CGT CTG TAT AGG ATC TCT GTA TA) with X=5'R- or 5'S-cdA or dA were obtained by analysis of the UV melting profiles. Typical UV melting profiles are shown in Figure 2 (panels a, b) and the melting points of the duplexes are summarized in Table 2. Both cdA lesions destabilize the 23-mer duplexes as shown by the differences in the melting points (T_m), ΔT_m=T_m (modified) – T_m (unmodified). The ΔT_m values are near -1 and -2 °C in the case of duplexes with either the 5'R-cdA or the 5'S-cdA lesions in the 23-mer duplex sequence context investigated (Table 2). Analogous destabilizations due to cdPu lesions have been observed previously in different sequence contexts and oligomer lengths [14,44,45].

Table 2. Melting points, T_m, of 23-mer DNA duplexes

5'-d(GCA GAC ATA TCC TAG AGX CAT AT)-3'	T _m , °C
3'-d(CGT CTG TAT AGG ATC TCT GTA TA)-3'	
X=dA (unmodified)	60.0±0,3
X=5'R-cdA	59.0±0,2
X=5'S-cdA	58.0±0,3

**Figure 2.** UV melting curves of 23-mer duplexes containing (a) 5'S-cdA lesion and (b) 5'R-cdA lesion.

UV melting curve of unmodified double stranded is shown in Figure S3.

3.3. PARP1 recognizes an undamaged dA and cdA diastereomeric lesions in ds-oligonucleotides

Employing circular dichroism (CD), we initially determined the secondary structure of PARP1, in the absence and presence of ds-oligonucleotides, by following the absorbance of the peptide bond in the "far-UV" spectral region 190-250 nm (Figure 3). The spectrum of each oligonucleotide was subtracted from that of the corresponding PARP1/oligo complex to illustrate the effects from the protein alone. Changes in CD signals resulting from the protein-DNA complex were used to determine the affinity of the PARP1-oligonucleotide interaction as well as to provide the information about the nature and conformational changes of PARP1 protein. In addition to the measurement of the differences in the trends of spectra, two parameters of protein CD spectra, the variability of negative ellipticity minimum and the possible wavelength shift of the minimum, were also measured [46].

The PARP1 binding to a double stranded (ds) oligonucleotide substrate without or with a 5'S- or 5'R-cdA (ds-N, ds-5'S, ds-5'R) was determined by incubating the substrates at 50 nM, 100 nM and 200 nM with PARP1 (0.13 nM). The changes in CD signals among the PARP1 alone and with oligonucleotide substrate establish the interaction between PARP1 and damage and undamaged DNA sequences. We have observed that this interaction was different in function of the substrate used, as shown by the profile of the CD spectra (Figure 3a-c) and by the different trend of the ellipticity minimum (Table 3).

The CD spectra showed that at almost all concentrations of the substrates, except for 200 nM ds-5'S substrate, the binding of PARP1 to the substrates led to a beta conformation of protein indicating that the substrates were able to stabilize the structure of PARP1 protein (Figure 3a-c, Table 3). Interestingly, we found that PARP1 interacted differently with the two diastereoisomeric lesions.

At 200nM only ds-5'R maintains rather unchanged the beta conformation of PARP1, which is highly stabilized. This result leads to hypothesize that the substrates may interact differently with the protein.

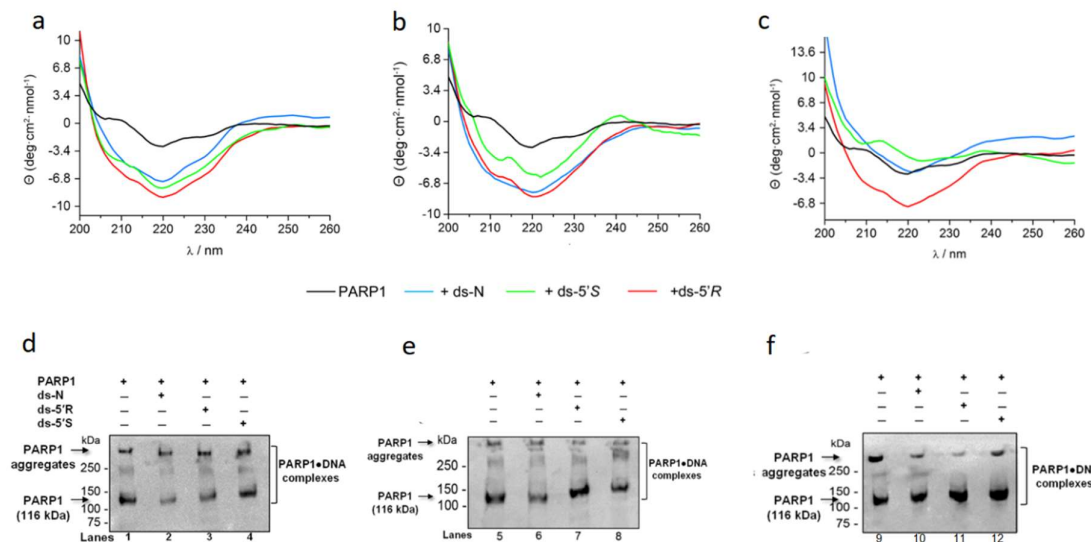


Figure 3. Circular dichroism and immunoblotting analysis for determining PARP1 binding to ds-oligonucleotides with cdA lesions. (a-c) The CD profiles of PARP1 without or with the substrates. Human PARP1 protein only (15 μ g mL⁻¹, black line), PARP1 along with 50 nM (a), 100 nM (b) or 200 nM (c) ds-N (blue line), or ds-5'S (green line)

or ds-5'R (*red line*) respectively. (d-f) Immunoblotting results of PARP1 in the presence of 50 nM (d), 100 nM (e) and 200 nM (f) oligonucleotide substrate. Lanes 1, 5 and 9 correspond to PARP1 only. Lanes 2, 6 and 10 correspond to PARP1 with 50 nM, 100 nM and 200 nM ds-N, respectively. Lanes 3, 7 and 11 correspond to PARP1 with 50 nM, 100 nM and 200 nM substrates containing a ds-5'R-cdA, respectively. Lanes 4, 8 and 12 correspond to PARP1 with 50 nM, 100 nM and 200 nM substrates containing a ds-5'S-cdA, respectively. The results in the panels d-f are from different immunoblottings; the full-length blots are shown in Figure S5.

The negative ellipticity minimum was very close for all substrates at 50 nM, but it exhibited a trend towards the positive values with increasing concentrations of all substrates (Table 3).

In case of the ds-5'R the substrate reduced the variation of negative ellipticity minimum much less in comparison with the other strands and resulted in the best arrangement of PARP1 spectrum (Figure 3 a-c, Table 3). It has been reported that PARP1 can interact with a DNA substrate without a lesion [47,48], while here, for the first time, we showed that PARP1 binds to both 5'R and 5'S diastereomeric cdA lesions.

Table 3. Ellipticity values, were converted into molar ellipticity Θ ($\text{deg}\cdot\text{cm}^2\cdot\text{nmol}^{-1}$) based on the molecular weight of PARP1 proteins, of human PARP1 alone and along with increasing concentrations (50nM, 100nM, 200nM) of double stranded oligonucleotides (ds-N, ds-5'S-cdA, ds5'R-cdA) respectively at 220 nm. The results are illustrated as mean values of triplicate with errors $\pm 5\%$.

Compounds	$\Theta_{220\text{nm}}$	$\Theta_{220\text{nm}}$	$\Theta_{220\text{nm}}$
	50nM	100nM	200nM
PARP1	-2.84	-2.84	-2.84
PARP1+ds-N	-7.14	-7.79	-2.46
PARP1+ds-5'S	-7.96	-5.82	-0.53
PARP1+ds-5'R	-9.09	-8.26	-7.26

To further evaluate PARP1 interaction with the substrates, an aliquot of the mixtures used for CD spectra was loaded on a SDS-polyacrylamide gel. PARP1 protein in the protein-oligonucleotide complex was immunoblotted and analysed by an anti-PARP1 antibody (Figure 3 d-f). The results showed that PARP1 protein alone exhibited a different mobility from PARP1-oligonucleotide complexes.

Since control ds-N and ds-5'S or -5'R oligonucleotides differ only for the cdPu lesions, any increase in PARP1 binding to the modified ligands, compared to control DNA, must be due to a better interaction of PARP1 with the lesions. In fact, the mobility of the protein-oligonucleotide complexes with a cdA lesion in the gel was retarded compared to N, and formation of complexes at higher molecular weight was detected (Figure 3d, lanes 2-4; Figure 3e lanes 6-8 and Figure 3f lanes 10-12). The broadening bands in the gels suggested the formation of various species of PARP1-oligonucleotide complexes with various intermediate conformations of PARP1 proteins.

DNA end binding with PARPs cannot be excluded. Nevertheless, the shift of enzyme-oligonucleotide complexes is different with each ligand. The results of immunoblotting analysis

confirmed the interaction between PARP1 and the different DNA substrates with or without cdA lesions.

In order to confirm these PARP mobility shift results, we examined the formation of the PARP1 binding to ds-N, ds-5'S, ds-5'R, by ^{32}P -labelled DNA gel mobility shift assay at various concentrations of PARP1 (30, 40 and 50 nM). This assay was useful to understand the behaviour of the DNA counterpart, confirming the recognition of all three substrates by PARP1 and the formation of PARP1-oligonucleotide complexes (Figure 4 and Figure S6 for full-length gel images). However, it is worth nothing that this assay has only a qualitative meaning, whereas it cannot appreciate significant differences in the binding of the undamaged and damaged substrates.

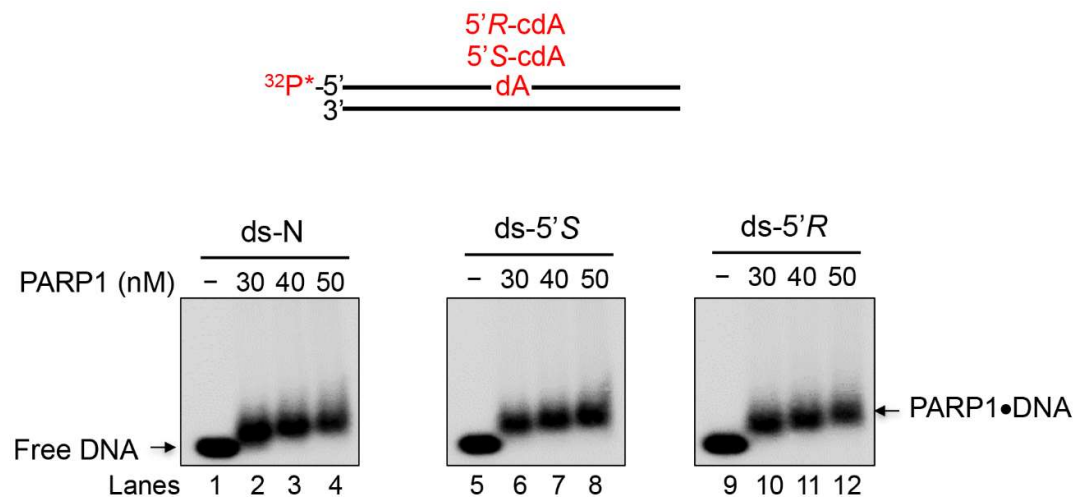


Figure 4. Gel mobility shift assay for determining PARP1 binding to ds-oligonucleotides with cdA lesions. Lanes 1, 5 and 9 correspond to the substrate only. Lanes 2, 6 and 10 correspond to binding mixture with 30 nM PARP1. Lanes 3, 7 and 11 correspond to binding mixture with 40 nM PARP1. Lanes 4, 8 and 12 correspond to binding mixture with 50 nM PARP1. The DNA substrates are schematically illustrated above the gel. Grouping of gels are cropped from different parts of the same gel. The original gel is represented in Figure S6. The gel mobility shift assay procedure is described in Materials and Methods in the main text.

3.4. PARP1 binding to undamaged dA and cdA lesions with different affinities

To further confirm the PARP1 binding to the substrates with or without cdA lesion, fluorescence spectroscopy was employed to determine the formation of PARP1-oligonucleotide substrate complex. The binding of the protein to the substrates was determined by measuring the quenching of intrinsic PARP1 fluorescence. This is because the intrinsic fluorescence of tryptophan residues in PARP1 proteins can be quenched by DNA bases when the protein forms a complex with a DNA substrate, that can also modulate the local environment of the fluorophore, such as the changes of hydrophobicity of the microenvironment. A hydrophobic environment increases the fluorescence intensity from a tryptophan, an aromatic and hydrophobic amino acid, whereas the fluorescence decreases if tryptophan moieties are exposed to a hydrophilic environment. Thus this method can also be used to determine the conformational change of PARP1 protein within its complex with DNA and the binding affinity of the protein to DNA. In Figure 5, fluorescence intensity of PARP1 in the

presence of ds-5'R substrate at 50 and 100 nM (panels a and b), overlapped that of PARP1 alone, although the maximum value tends to shift towards a longer wavelength (lower frequency). This indicated that the binding of PARP1 to the substrates did not alter significantly the hydrophobic environment of the fluorophore Trp, although inducing a change by increasing the wavelength of maximal intensity (blue shift). The decrease of fluorescence intensity appeared at 200 nM ds-5'R substrate (Figure 5c). Thus, on oligo binding, perturbation of Trp environment was limited, but enough to allow PARP1 conformational change. In fact, these results are in line with those of Figure 3 (a-c), where the arrangement of PARP1 secondary structure to get a beta conformation took already place at 50 nM and 100 nM ds-5'R substrate, and was stable up to 200 nM ds-5'R substrate.

For ds-5'S at a low concentration, 50 nM (Figure 5a), PARP1 exhibited a similar binding to the ds-5'R substrate. At a high concentration of N, S substrates (Figure 5c), the binding of PARP1 led to exposure of the fluorophores to a more hydrophilic environment, with a decreased fluorescence intensity, and a high perturbation of Trp surrounding. In Figure 3b, CD spectra show that ds-5'S and N substrates are less efficient than ds-5'R to stabilize PARP 1 beta-conformation.

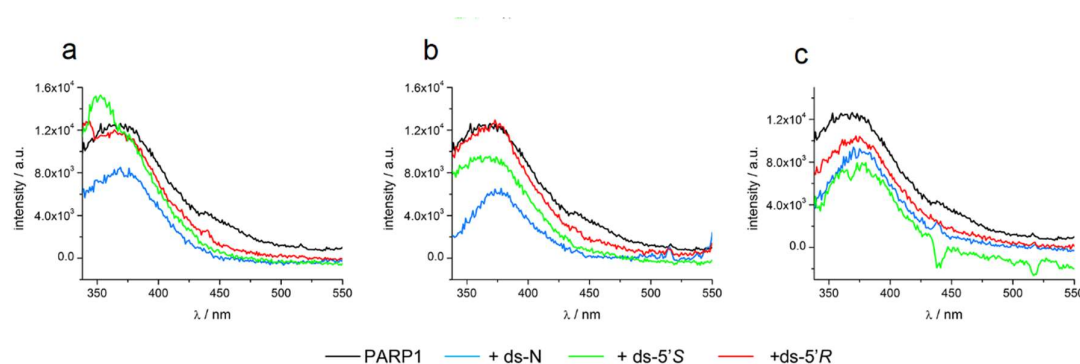


Figure 5. Intrinsic fluorescence of PARP1 in the absence and presence of DNA substrates.

(a-c) The fluorescence profiles of PARP1 protein alone ($15\mu\text{g}\cdot\text{mL}^{-1}$, black line) or that of PARP1 along with 50 nM (a), 100 nM (b), 200 nM (c) of ds-N (blue line) or ds-5'S (green line) or ds-5'R (red line).

The intrinsic fluorescence intensity altered by the formation of PARP1-oligonucleotide complex allowed us to calculate the affinity constants (K_a) of PARP1 binding to different substrates with a cdA lesion at a saturating concentration of the substrates, which are shown in Table 4.

Table 4. Affinity constants (K_a) of PARP1 binding to substrates.

Substrates	Saturation at (nM)	K_a^* (M^{-1})
ds-N	100	$1.51\cdot 10^7$
ds-5'S	200	$3.19\cdot 10^9$
ds-5'R	200	$1.21\cdot 10^7$

*Details of constant calculations at a saturating concentration of substrates are illustrated in Supporting Information (pages S7 and S8).

The results showed that PARP1 bound to all, damaged and undamaged, substrates with the same affinity at a saturating concentration (10^7 order of magnitude), except for ds-5'S where is two orders of magnitude higher than the others (10^9 order of magnitude).

By considering that both damaged substrates reach saturation at 200 nM and induce a qualitatively comparable conformational change of PARP1, it is conceivable that the binding affinity of ds-5'S might be due to a different way of interaction with the protein.

To further explore the role of the zinc finger domain of PARP1 in mediating its binding to the undamaged and damaged substrates with cdA lesions, we examined the binding of mouse PARP2 to the substrates. Since PARP2 does not have a zinc finger, the difference of the substrate binding of PARP1 and PARP2 would indicate if the zinc finger of PARP1 is involved in the substrate binding and recognition of the cdA lesions. It should be noted that both PARP1 and PARP2 have two C-terminal domains—Trp-Gly-Arg (WGR) and catalytic (CAT) regions. The N-terminal region (NTR) of PARP1 contains over 500 amino acid residues with four regulatory domains, including three zinc-fingers that are essential for DNA-dependent PARP1 activity, whereas PARP2 has a small NTR (70 residues) and lacks the zinc fingers. However, the region is enriched of basic amino acid residues that may serve as a potential DNA binding domain [49,50]. A previous study has shown that the basic amino acid residues within the NTR of this protein exhibited potential DNA-binding ability [48].

However, recent structural and biochemical studies have shown that WGR domain of PARP1 plays a central role in coordinating with allosterical regulation of the CAT domain as well as with Zn1 and Zn3 domains [51,52]. Since a recent study from the Pascal group suggests that PARP2 can be activated in response to specific DNA repair intermediates, such as the nicked DNA [29], we then explored the binding of mouse PARP2 to the undamaged DNA substrate and the substrates containing a cdA lesion (Figure S4). The results of fluorescence and CD spectroscopy showed that the binding of PARP2 to the 5'R-cdA diastereomer resulted in an increase of fluorescence intensity and the blue shift of negative ellipticity minimum wavelength, indicating that 5'R-cdA isomer stabilizes the fluorophore hydrophobic environment. This further suggests that the lesion stabilized the conformation of PARP2, an unexpected indication as it is well considered that PARP2 NTR is disordered. The last evidence strongly suggests that PARP2 interact with the 5'R-cdA in its native conformation. In contrast, opposite results were obtained from the binding of PARP2 to a 5'S-cdA lesion revealing that PARP2 bound to 5'S-cdA in a different manner from a 5'R diastereomer.

The obtained diverse PARP2 interaction with diastereomeric cdA lesions suggest that the zinc finger domain of PARP1 is not required for sustaining its complex with a substrate containing a cdPu lesion. This has further paved the way for further studies on the function of mutant forms of PARP1 or different PARP domains in mediating the binding of PARP proteins with DNA substrates.

4. Conclusions

In this study we characterized the binding affinity and recognition patterns by the PARP1 protein of 5'S-cdA and 5'R-cdA lesions, unique DNA adducts associated with the NER pathway, in double stranded oligonucleotide substrates. It must be underlined that the presented results help to understand the structural interactions of PARPs with modified oligonucleotides, which is the novelty of this work, but further studies are required for proposing any functional role of these enzymes. Our finding for the first time revealed the ability of PARP1 to recognize *in vitro* the peculiar DNA backbone distortion and differentiate the diastereomeric forms of a cdA lesion, as detected by

different approaches at distinct oligonucleotide sequences, with higher affinity constant for the 5'S lesion in a mimetic model of ds DNA.

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