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Title Page

ATP Modulates Poly(ADP-ribose) Polymerase-1-Facilitated Topoisomerase I-linked DNA Religation in the Presence of Camptothecin

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Running Title Page

Running Title: ATP Modulates Topoisomerase-I-Linked DNA Regliation

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PARP-1, poly(ADP-ribose) polymerase-1; TLD, topoisomerase I-linked DNA.

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ABSTRACT

Poly(ADP-ribose) polymerase-1 (PARP-1) was reported to promote the religation activity of topoisomerase I in the presence of camptothecin by itself through the direct interaction with topoisomerase I or by the formation of poly(ADP-ribosyl)ated topoisomerase I. We have previously demonstrated that ATP inhibited PARP-1/NAD-facilitated religation of topoisomerase I-linked DNA (TLD) in the presence of camptothecin. The mechanism of action was further studied in the present work. ATP as well as other nucleotides, including CTP, UTP and GTP, had no effect on topoisomerase I cleavage and religation activities in the absence of camptothecin. In the presence of camptothecin or its derivative, topotecan, ATP (at up to 2 mM) inhibited PARP-1/NAD-facilitated TLD religation in a dose-dependent manner. This could be due to the suppression of topoisomerase I poly(ADP-ribosyl)ation through the competition with NAD for the binding site(s) on PARP-1. The interaction between ATP and PARP-1 was independent of ATP hydrolysis. Study of different nucleotide analogs revealed that the structure could determine the dose response of nucleotides. Besides, it was noted that higher concentrations of ATP and CTP (at 2.5 mM or higher) promoted DNA religation by a PARP-1-independent mechanism. Our study implies the possible role of ATP and other nucleotides in the regulation of topoisomerase I activity in the presence of camptothecin analogs.

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INTRODUCTION

Human topoisomerase I is one of the key enzymes that control the topological state of DNA related to DNA replication, transcription and recombination (Champoux, 2001; Pourquier and Pommier, 2001; Wang, 1985, 1996, 2002). Relaxation of DNA by topoisomerase I involves four catalytic steps: (a) DNA binding; (b) cleavage with the formation of topoisomerase I-linked DNA (TLD) complex; (c) strand rotation; and (d) religation. Once topoisomerase I binds to the DNA, it creates a transient DNA break, by the formation of a covalent tyrosyl phosphodiester bond with the 3'-end of DNA. After the DNA is relaxed, topoisomerase I religates the cleaved DNA. This cleavage-religation coupling, which is important for cell function, can be disrupted by topoisomerase I poisons, such as camptothecin and topotecan (Lorence and Nessler, 2004; Pommier, 2006; Sriram et al, 2005; Thomas et al, 2004). Even though stabilization of the TLD complex, induced by camptothecin, is not sufficient to cause cell death, the stabilized TLD has the potential of being converted to double strand DNA breaks, which can lead to cell death (Hsiang et al, 1989; Liu et al, 2000; Wu and Liu, 1997). The religation of TLD is therefore critical in the process.

PARP-1 is a highly conserved nuclear enzyme that plays diverse roles in many molecular and cellular processes, including DNA damage detection and repair, chromatin modification, transcription, cell death pathways, insulator function, and mitotic apparatus function (Burkle et al, 2005; Ivana and Diederich, 2004; Kim et al, 2005; Meyer-Ficca et al, 2005; Oei et al, 2005; Strosznajder et al, 2005). It has been known to modulate the toxicity of camptothecin by catalyzing the polymerization of ADP-ribose units from NAD molecules to target proteins, including topoisomerase I and PARP-1 (Beidler et al, 1996;

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Burkle, 2005; Chatterjee et al, 1989). Previously, we reported that PARP-1 could destabilize the TLD complex, and hence promote religation either by direct interaction with topoisomerase I or by the formation of poly(ADP-ribosyl)ated topoisomerase I in the presence of NAD. Furthermore, ATP was found to be a potential regulator for the camptothecin action by inhibiting the activity of PARP-1/NAD on topoisomerase I (Park and Cheng, 2005).

The effect of ATP and other nucleoside analogs on topoisomerase I activity has been studied (Castora and Kelly, 1986; Chen and Castora, 1988; Chen and Hwang, 1999; Foglesong and Bauer, 1984; Goto et al, 1984; Liu et al, 1989; Low and Holden, 1985; Rowe et al, 1981; Sekiguchi and Shuman, 1994; Shaffer and Traktman, 1987; Stewart et al, 1996). ATP inhibited the activity of topoisomerase I from human leukemia cells (Castroa and Kelly, 1986; Liu et al, 1989), Hela cells (Low and Holden, 1985) and *Ustilago maydis* (Rowe et al, 1981). However, ATP did not inhibit the activity of topoisomerase I from yeast (Goto et al, 1984) at concentrations up to 5 mM. Foglesong *et al*. and Sekiguchi *et al*. demonstrated that ATP could promote the DNA relaxation activity of topoisomerase I from vaccinia virus (Foglesong and Bauer, 1984; Sekiguchi and Shuman, 1994), whereas, Shaffer and Traktman (1987) observed that the activity of topoisomerase I, isolated from vaccinia virus cores, was inhibited by ATP. Despite these conflicting observations, it is generally agreed that the DNA relaxation activity can be inhibited by ATP.

The mechanism of the inhibitory activity is unclear. Stewart *et al*. claimed that ATP inhibits topoisomerase I activity by lowering the free Mg^{2+} concentration, which is essential for the enzyme activity (Stewart et al, 1996). However, Chen and Hwang

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reported that the inhibition of topoisomerase I activity was Mg^{2+}-independent (Chen and Hwang, 1999). ATP was also shown to have direct impact on topoisomerase I and PARP-1. ATP inhibits PARP-1 auto-poly(ADP-ribosyl)ation (Kim et al, 2004; Kun et al, 2004), as well as poly(ADP-ribosyl)ation of DNA-PKcs (Ariumi et al, 1999) and topoisomerase I (Park and Cheng, 2005). An ATP binding site was identified within the C-terminal domain of topoisomerase I for its kinase activity (Rossi et al, 1996, 1998), even though topoisomerase I does not need ATP as a cofactor or an energy source for its DNA relaxation activity. However, the role of the direct interaction between ATP and topoisomerase I in the regulation of DNA relaxation activity is still unknown.

In the present study, we investigated the mechanism of action of ATP on PARP-1/NAD facilitated-TLD religation. Our data reveals the possible role of ATP in the regulation of topoisomerase I activity, in the presence of camptothecin and topotecan, through the direct interaction with PARP-1 and topoisomerase I.

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MATERIALS AND METHODS

Drugs and Compounds. Camptothecin was provided by Dr. Zong-Chao Liu of the Cancer Institute at Sun Yat-Sen University of Medical Sciences (Guangzhou, China). Topotecan was purchased from GlaxoSmithKline (Uxbridge, Middlesex, UK). ATP, UTP, CTP, GTP, ADP, AMP, Ap3A, Ap4A, Ap5A, CDP-choline and trypsin were acquired from Sigma Chemical Co. (St. Louis, MO) and dATP, TTP, dCTP, dGTP and AMP-PNP were obtained from Roche Applied Science (Indianapolis, IN). ddATP, ddTTP, ddCTP and ddGTP were acquired from Amersham Bioscience (Pittsburgh, PA). 2′-Ara-ATP was purchased from TriLink (San Diego, CA). All nucleotides were prepared in 50 mM Tris-HCl, (pH 8.0) and mixed with an equal amount of $MgCl_2$ (molar ratio: 1 to 1).

Recombinant Proteins. Full-length human topoisomerase I was expressed in Sf-9 cells and was purified as described previously (Park and Cheng, 2005). Human recombinant PARP-1 was purchased from Trevigen (Gaithersburg, MD).

Oligonucleotides. Oligonucleotides were synthesized by IDT DNA (Coralville, IA) and the sequences are as follow:

ON4: 5′-pTAAAAATTTTTCCAAGTCTTTTTTC-3′

ON5: 5′-GAAAAAAGACTTGG-3′

ON6: 5′-GGAAAAATTTTTA-3′

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ON5 was 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly MA) and annealed to ON4 to generate a duplex oligonucleotide substrate, as described before (Park and Cheng, 2005).

Oligonucleotide Cleavage Reaction. Topoisomerase I (220 fmols) and radio-labeled ON5-ON4 oligoduplex (~50 fmols) were incubated in the presence or absence of nucleotide in a 10 μl reaction containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1mM EDTA, and 30 μg/mL bovine serum albumin. The reaction was performed at 37°C for 15 min and stopped by adding SDS to the final concentration of 0.5%. Covalent-linked topoisomerase I was digested by trypsin (0.2 mg/mL final concentration) at 37°C for 30 min. and 5 μl of loading buffer (98% formamide, 10 mM EDTA, 10 mM NaOH, 0.1% bromphenol blue, and 0.1% xylene cyanol) was added, boiled and separated on a 20% denaturing urea/polyacrylamide gel electrophoresis. The gel was analyzed by autoradiography and PhosphoImaging screen (Molecular Dynamics-Amersham Bioscience, Piscataway, NJ). Alternatively, reactions were stopped by adding Laemmli loading buffer without digestion. Covalent complexes of topoisomerase I with cleaved oligonucleotides were separated from the uncleaved substrates by SDS-PAGE and visualized by autoradiography.

Oligonucleotide Religation Reaction. Religation reaction was initiated with the suicide cleavage complex, generated by a cleavage reaction by adding 5 pmoles of ON6 alone or with components such as PARP, NAD or nucleotides. The reaction was performed at

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37°C for 30 min and analyzed by 20% urea/polyacrylamide gel electrophoresis, as described above, except that TLD was digested by 1 mg/mL proteinase K at 37°C for 1 h.

Plasmid Relaxation Assay. The reactions were carried out in a buffer containing 0.25 μg supercoiled pUC18, 220 fmol topoisomerase I, 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1mM EDTA, 5% glycerol, and 30 μg/mL bovine serum albumin. The reactions were incubated for 30 min at 37°C and terminated by the addition of 1% SDS. DNA was then separated in a 1% agarose gel with or without ethidium bromide (0.25 μg/ml). To study the conversion of nicked DNA to the relaxed form by PARP-1/NAD, the reactions were performed in the presence of 40 μM camptothecin prior to the addition of PARP-1 (21.5 fmols) and NAD (18.2 μM). The reactions were then continued for another 30 min at 37°C.

Immunoprecipitation Assay. Religation reactions were performed, followed by the incubation with anti-PAR antibody (Trevigen, MD) and protein G plus/protein A agarose (Calbiochem, San Diego, CA). Supernatants were collected and immunoprecipitates were washed five times with 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1mM EDTA, 150 mM NaCl, and 1% Triton X-100. Samples were electrophoresed on 7.5% acrylamide gel and analyzed by western blot analysis using anti-topoisomerase I antibody (clone 21, generated by our lab), as described previously (Park and Cheng, 2005).

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RESULTS

Nucleoside triphosphates do not inhibit topoisomerase I cleavage activity

Relaxation of DNA involves DNA cleavage at specific sites with the formation of a covalent topoisomerase I-DNA adduct. The direct impact of nucleoside triphosphates on these processes was studied using a 14-mer/25-mer oligonucleotide duplex (ON4/ON5), which has only two nucleotides downstream of the topoisomerase I cutting site, in order to prevent religation to occur and allow the formation of the suicide cleavage complex (TLD in the absence of camptothecin). The TLD product was digested with trypsin in order to be size-fractionated by polyacrylamide gel electrophoresis. Due to incomplete digestion of TLD (Park and Cheng, 2005; Stewart et al, 1999), cleavage products (12-mer oligonucleotides containing amino acid residues of topoisomerase I) migrated slower than the substrate (14-mer). Figure 1A showed that ATP, UTP, CTP and GTP, up to concentrations of 4 mM, did not have a significant effect on the cleavage process.

We also studied the impact of nucleoside triphosphates on the formation of TLD. Instead of digesting with trypsin, the covalent complexes of topoisomerase I with cleaved oligonucleotides were separated from the uncleaved substrates by SDS-PAGE. Fig. 1B showed that the nucleoside triphosphates did not interfere with the TLD formation. Our data, therefore, suggest that the four nucleoside triphosphates have no effect on the DNA binding and cleavage activities of topoisomerase I.

Impact of nucleoside triphosphates on topoisomerase I religation activity

Following DNA cleavage, relaxation of DNA by topoisomerase I is known to be completed by the religation process. The effect of nucleoside triphosphates on

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topoisomerase I-facilitated DNA religation was studied by the addition of a complementary 13-mer (ON6) to the suicide cleavage complex, which was obtained in the cleavage reaction. ON6 quickly religated with ON5 (12-mer) in the presence of topoisomerase I and resulted in the formation of the 25-mer religation product (Fig 2A). The four nucleoside triphosphates, ATP, UTP, CTP and GTP, at up to 4 mM concentration, did not interfere with the TLD religation process (Fig 2B).

Camptothecin and topotecan, two topoisomerase I poisons that are used clinically for cancer treatment, inhibited the religation process by 90% (Fig. 2C). Interestingly, the suppressed religation activity of topoisomerase I was reversed by ATP and CTP, but not by UTP and GTP, at concentrations of 4 mM or higher. This reversal effect started to become apparent at 2.5 mM (Fig. 2D).

Inhibition of PARP-1/NAD-facilitated religation action of topoisomerase I by ATP in the presence of camptothecin does not require ATP hydrolysis

We have previously demonstrated that PARP-1/NAD-facilitated TLD religation in the presence of camptothecin, while ATP reversed the action of PARP-1/NAD (Park and Cheng, 2005). Relaxation assay revealed that ATP could inhibit the PARP-1/NAD-mediated religation of camptothecin-induced nicked plasmid DNA (Fig. 3A). Camptothecin stabilized the TLD complex and resulted in the formation of nicked DNA, which was religated in the presence of PARP-1/NAD (Fig. 3A, lower panel). Increase of nicked DNA level was observed with up to 2 mM of ATP in a dose-dependent manner. This is consistent with our previous finding that ATP inhibited the action of PARP-1/NAD. In the oligonucleotide religation assay, 2 mM of ATP was found to suppress up

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to 90% of the religation activity of TLD in the presence of PARP-1/NAD and camptothecin analogs (Fig. 3B, upper panel). To examine whether ATP hydrolysis was required for the inhibition of PARP-1/NAD-facilitated topoisomerase I religation activity, the impact of AMP-PNP, which is an ATP analog that prevents hydrolysis of the phosphate bond, was studied. Fig. 3B (lower panel) indicated that AMP-PNP could also inhibit the PARP-1/NAD action at a relatively lower potency, when compared with ATP. This suggests that ATP hydrolysis is not essential for the inhibitory activity of ATP. The structure, however, could be a critical factor that determines the potency of nucleotides. The effect of ATP and AMP-PNP was found to be biphasic. Regaining of religation activity was observed at higher concentrations of nucleotides (> 3 mM and > 8 mM of ATP and AMP-PNP, respectively).

ATP suppresses poly(ADP-ribosyl)ation of topoisomerase I and inhibits PARP-1/NAD-facilitated TLD religation activity in a competitive manner

In the previous study, our data have indicated that poly(ADP-ribosyl)ation could play an important role in promoting TLD religation activity (Park and Cheng, 2005). To investigate whether ATP inhibits poly(ADP-ribosyl)ation of topoisomerase I, religation reaction was performed with cold (unlabeled) ON6 13-mer in the presence of ATP. Anti-PAR immunoprecipitate, as well as the supernatant collected before and after the religation reaction, were probed with topoisomerase I antibody. The result showed that poly(ADP-ribosyl)ation of topoisomerase I decreased in a dose-dependent manner with increasing ATP at concentrations up to 4 mM (Fig. 4A). This suggests that suppression of PARP-1 activity by ≤ 2 mM of ATP may account for, at least in part, the inhibitory

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effect on the religation. Low level of poly(ADP-ribosyl)ation was detected in the presence of 0.4 mM ATP, at which TLD religation was drastically inhibited (Fig. 3). This indicates that suppression of TLD religation may not require complete blockage of PARP-1 activity. Interestingly, poly(ADP-ribosyl)ation of topoisomerase I was found to be almost completely suppressed by 4 mM of ATP, at which we observed an increase in TLD religation activity (Fig.3). This implies that ATP at higher concentrations could promote the religation process by a different mechanism that is independent of PARP-1.

To understand the nature of ATP inhibition on PARP-1/NAD-facilitated TLD religation activity, a kinetic analysis was performed by running religation reactions with various concentrations of ATP in the presence of camptothecin, PARP-1 and various concentrations of NAD at 37°C for 30 min. The double-reciprocal plot showed that ATP dose-dependently decrease *K*m *app*, but has no effect on *V*max, suggesting that ATP is a competitive inhibitor of PARP-1 with respect to NAD for the facilitation of topoisomerase I religation in the presence of camptothecin (Fig. 4B). The *K*m value of the PARP-1/NAD action on topoisomerase I religation was 7.7 ± 1.5 μM. Re-plotting *K*m *app* against $[ATP]^2$ revealed a linear relationship where the *K*i *app* value was found to be 90 ± 6.0 μM.

Inhibition on PARP-1/NAD-facilitated topoisomerase I religation by nucleotides could be structure-specific

Although hydrolysis of ATP is not essential for its inhibitory activity, the chemical structure seems to determine the potency of the inhibition on TLD religation activity facilitated by PARP-1/NAD. Comparison of nucleotide analogs with different number of

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phosphate group revealed that both ATP and ADP were almost equally potent against the religation (EC_{50} ~ 0.25 mM and ~ 0.3 mM, respectively), while AMP exhibited the weakest inhibition (EC_{50} ~ 2 mM). The two phosphate groups could therefore be critical for the inhibitory activity of ATP.

The importance of the sugar moiety of nucleotides was examined by comparing ATP, dATP, ddATP and 2′-ara-ATP, which has an arabinose instead of a ribose. The religation activity was found to be less susceptible to the action of 2'-ara-ATP (EC_{50} ~ 1 mM) (Fig. 5B). The presence of the deoxy- or dideoxy- ribose moiety in dATP or ddATP caused a significant decrease in the potency of inhibition (EC_{50} ~ 1.8 mM and 1.6 mM, respectively), when compared with ATP. The result suggests that the structure of the sugar moiety is critical for the inhibition.

Assessment of nucleotide analogs with different bases showed that ATP and CTP were stronger inhibitors (Fig. 5C) than GTP and UTP. This illustrates the importance of the base for the inhibition. Although no significant difference in potency was observed among various deoxynucleoside and dideoxynucleoside triphosphates (Fig. 5D and E), the corresponding ribonucleotides appeared to be significantly stronger inhibitors, suggesting that the hydroxyl group(s) on the 2’ or 3’ position of the ribose could play an important role in recognition.

Three naturally occurring diadenosine polyphosphates, Ap3A, Ap4A and Ap5A, were also found to inhibit religation, while no significant difference in potency was observed among the three analogs (Mclennan, 2000; Ogilvie et al, 1996) (Fig. 5F). Their EC_{50} values fell within the concentration range from 0.6 to 1.0 mM. A maximum inhibition of

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up to 60% on the religation activity was observed with these compounds at concentrations of 2 mM or higher (data not shown).

Since CTP showed strong activity comparable to that of ATP, several cytidine analogs were examined. CDP-choline (cytidine 5'-diphosphocholine) is a naturally occurring metabolite, which has a choline group instead of a γ-phosphate and is essential for the synthesis of phosphatidylcholine, one of the cell membrane components (Fioravanti and Yanagi, 2005). CDP-choline inhibited PARP-1/NAD action on the religation activity of topoisomerase I in the presence of camptothecin at a lower potency (EC_{50} ~ 1.5 mM) compared with CTP (EC_{50} ~ 0.3 mM) (Fig. 5G). Decrease in the potency of diadenosine polyphosphates and CDP-choline, when compared with ATP and CTP, respectively, indicates that modification of the terminal phosphate group may interfere with the interaction between nucleotides and PARP-1. In addition, metabolites of two anti-cancer nucleoside analogs, gemcitabine triphosphate, which has two fluorines in the 2' position of the ribose, and troxacitabine triphosphate, which is an L-configuration nucleoside with a dioxolane sugar moiety, did not show significant effect on the religation (1% and 3% inhibition, respectively) at a concentration of 0.4 mM.

Taken together, our findings suggest that the inhibition of PARP-1/NAD-facilitated TLD religation by nucleotides at up to 2 mM could be structure-specific. The base, the sugar and the phosphate group could be critical for the action.

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DISCUSSION

Topoisomerase I is a well-known target of camptothecin analogs, which stabilize the TLD complex and may lead to double-strand breaks. We have previously shown that PARP-1 could facilitate the religation of TLD inhibited by camptothecin and ATP was identified as one of the important cellular factors that regulate the process (Park and Cheng, 2005). The present study demonstrates the multiple actions of ATP on the religation of TLD by interacting with PARP-1 and topoisomerase I.

Poly(ADP-ribosyl)ated topoisomerase I was reported to exhibit lower DNA relaxation activity (Ferro et al, 1983; Jongstra-Bilen et al, 1983). Our previous findings (Park and Cheng, 2005) as well as the present study, however, provide the evidence for the enhancement of the TLD religation activity by the modification. This indicates that poly(ADP-ribosyl)ation could lower the binding affinity of topoisomerase I to the DNA. This could account for the low relaxation activity of the purified poly(ADP-ribosyl)ated topoisomerase I observed by others. However, once the TLD complex is formed, poly(ADP-ribosyl)ation of topoisomerase I could facilitate the disassembling of topoisomerase I from the DNA and hence allow religation to occur.

In the absence of PARP-1/NAD, ATP inhibition of DNA relaxation activity has been well known, as reported by us and by others previously. However, it is not clear which catalytic step(s) is inhibited. In this study, we showed that ATP had no effect on oligonucleotide cleavage and religation activities of topoisomerase I. Our finding is consistent with the report by Chen and Castora (1988), who observed that ATP did not interfere with the binding and cleavage activity of topoisomerase I. We postulate that

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among the four catalytic steps of topoisomerase I, it is the strand rotation step that serves as the target for ATP (Castora and Kelly, 1986; Rowe et al, 1981).

In the presence of PARP-1/NAD, ATP inhibits TLD religation by competing with NAD for PARP-1 binding and thus suppresses poly(ADP-ribosyl)ation of topoisomerase I, as well as auto-poly(ADP-ribosyl)ation of PARP-1. This may lead to a decrease in the religation activity of TLD (Kun et al, 2004; Park and Cheng, 2005). It was reported that the mode of inhibition by ATP was non-competitive with respect to NAD in auto-poly(ADP-ribosyl)ation of PARP-1 (Kun et al, 2004). This indicates the possibility of the presence of two ATP binding sites of similar binding affinity on PARP-1, which is supported by our finding that the *K*m *app* is directly proportional to the square of the ATP concentration. However, it is also possible that conformational changes of topoisomerase I, induced by ATP, could make topoisomerase I more resistant to poly(ADP-ribosyl)ation by PARP-1/NAD. This mechanism cannot be ruled out at this time.

The interaction of nucleoside triphosphates with PARP-1/NAD could be structure-specific. Changes of the base, the sugar or the phosphate group of the nucleotide modulate the potency of the TLD religation activity. The activity of topoisomerase I appears to be more susceptible to the action of nucleotide triphosphates with the NH_2 group on the bases (i.e. ATP and CTP). In addition, nucleotides with two or more phosphate groups (i.e. ADP and ATP) were found to have a more potent effect on the religation activity of topoisomerase I. We suspect that nucleotides that are structurally similar to NAD (which possesses two phosphate groups and the NH_2 group on the

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nucleoside) could be stronger competitors with respect to NAD for the binding site(s) on PARP-1.

Direct interaction of topoisomerase I with ATP has been reported to be critical for its activity (Rossi et al, 1996, 1998). Besides the inhibitory effect of the nucleotide analogs described above, we also observed an additional effect on the religation activity of topoisomerase I. ATP and CTP, at 4 ~ 10 mM, reversed the inhibitory effect on the TLD religation activity in the presence of camptothecin analogs. The detailed mechanism of action by which ATP and CTP destabilize the TLD complex is not clear. Since HPLC analysis showed that ATP did not affect the stability of camptothecin (data not shown), the reversal action observed here could be due to the facilitation of topoisomerase I religation activity. It is conceivable that conformational changes of topoisomerase I, induced by ATP or CTP, could lead to the increase of DNA religation in the presence of camptothecin analogs. Direct interaction of ATP with topoisomerase I was shown to be a prerequisite for the phosphorylation of the SR protein by topoisomerase I (Rossi et al, 1996, 1998). It is reasonable to postulate that all the nucleotides could interact with topoisomerase I through the same ATP binding site, however, it is also possible that topoisomerase I has additional binding sites for the nucleotide. The binding of nucleotides to topoisomerase I could be structure-specific.

Desensitization of camptothecin inhibitory action on the religation activity of topoisomerase I by ATP and CTP, but not by UTP and GTP, revealed that the NH_2 group of adenine or cytosine could be required in the binding of nucleoside triphosphates to topoisomerase I in the promotion of TLD religation. In addition, ADP and AMP, at up to 4 mM concentrations, did not increase the religation activity of topoisomerase I in the

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presence of camptothecin (data not shown). It is likely that the presence of three phosphates, instead of two or less phosphates, is critical for the reversal action. This also implies that different ATP binding sites could be responsible for the differential impacts on the religation process.

Modulation of PARP-1 has been suggested to be a strategy to potentiate the chemotherapeutic action of topoisomerase I poisons (Chatterjee et al, 1989; Cimmino et al, 2007). We anticipate that nuclear ATP, as well as several naturally occurring nucleoside analogs, could play a key role in determining the effectiveness of camptothecin analogs in causing DSB, which leads to cell death. In essence, the alteration of intracellular ATP levels could interfere with PARP-1 activity and results in cells displaying different sensitivity to this class of topoisomerase I poisons. It is noted that the nuclear concentration of ATP, which is similar to that in the cytosol, is in the range of 1 ~ 10 mM (Gajewski et al, 2003; Jiang et al, 1998; Miller and Horowitz, 1986; Smith et al, 2005). Based on our in vitro findings, at these concentrations, PARP-1 activity was supposed to be inhibited by ATP in a competitive manner. The presence of the intrinsic PARP-1 activity in cells indicates that the regulation is likely dependent on the relative ratio of NAD/ATP, instead of the ATP pool size, because ATP is a competitive inhibitor with respect to NAD. In addition, ATP compartmentation may play a role in the regulation of PARP-1.

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Footnotes

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FIGURE LEGENDS

Fig. 1. The impact of nucleoside triphosphates on topoisomerase I (Topo I) cleavage activity. The cleavage reaction was done using 220 fmol of topoisomerase I in the presence and absence of nucleoside triphosphates (NTP). The cleavage activity was evaluated by measuring the amount of cleavage products (A) and the formation of TLD (B). Data were quantified using a densitometer.

Fig. 2. The impact of nucleoside triphosphates on TLD religation. (A) The religation reaction containing 220 fmol of topoisomerase I was performed as described under "Materials and Methods." The religation activity was evaluated by measuring the religation product formation in the absence (B) and presence (C and D) of 40 μM of camptothecin (CPT) or topotecan (TPT). The figures are representative of at least three independent experiments. Data were quantified using a densitometer.

Fig. 3. (A) The impacts of ATP on topoisomerase I (Topo I) relaxation of supercoiled plasmid DNA (upper) and the religation of camptothecin (CPT)-induced nicked DNA (lower) were studied in the absence and presence of ethidium bromide (EB), respectively. (B) The impact of ATP and AMP-PNP on the religation activity of TLD in the presence of PARP-1/NAD and camptothecin (CPT) or topotecan (TPT). The religation activity was determined in the presence of 21.5 fmols of PARP-1, 18.2 μM of NAD, 40 μM of camptothecin or topotecan and various concentrations of ATP (upper) or AMP-PNP (lower). The effect of

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AMP-PNP on topoisomerase I was examined in the absence of PARP-1/NAD (lower left). The religation reaction was performed as described under "Materials and Methods." The figures are representative of three independent experiments. Data were quantified using a densitometer.

Fig. 4. Mechanism of ATP inhibition. (A) The status of topoisomerase I poly(ADP-ribosyl)ation in the religation reaction containing 220 fmol of topoisomerase I, 21.5 fmol of PARP-1 and 18.2 μM NAD in the presence of ATP was examined as described. PAR immunoprecipitate (IP) and the supernatant collected before and after the religation reaction (Input) were immunoblotted with anti-topoisomerase I antibody. C represents the complete religation reaction containing protein G plus/protein A agarose without ATP and anti-PAR. (B) Kinetic analysis of ATP inhibition on PARP-1/NAD action. Religation reaction with 220 fmol of topoisomerase I and 21.5 fmol of PARP-1 was done at various concentrations of NAD and ATP. The double-reciprocal plot shows that the inhibitory action of ATP is in competition with NAD for PARP-1/NAD action. The square of the *K*i value was determined by plotting *K*m app under various concentrations of ATP. The data are illustrative of three independent experiments.

Fig. 5. Structure-activity relationship. The impact of ATP analogs (*A* and *B*), nucleoside triphosphates (*C*), deoxynucleoside triphosphates (*D*), dideoxynucleoside triphosphates (*E*), diadenosine polyphosphates (*F*) and CTP

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analogs (*G*) on the religation activity of topoisomerase I, in the presence of 21.5 fmols of PARP-1, 50 μM of NAD and 40 μM of camptothecin. The religation reaction was performed as described. The graphs show the religation product expressed as % of PARP-1/NAD control (% of religation product with nucleotide/% of religation product without nucleotide × 100). The data present means ± SEM from three independent experiments.

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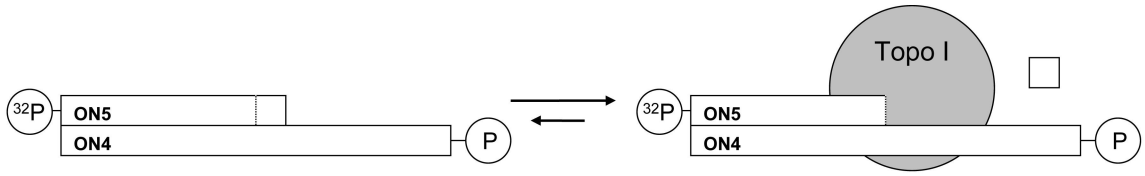
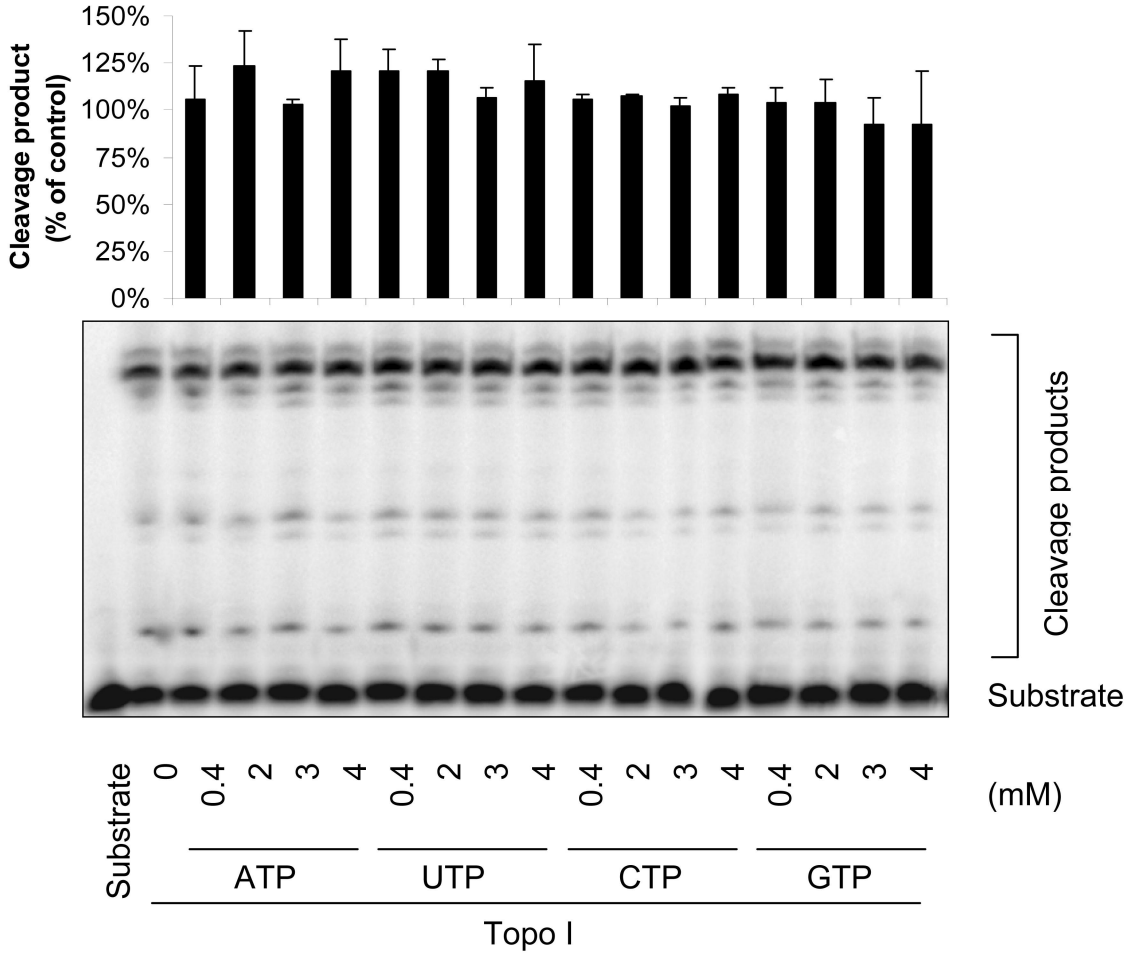
Figure 1



A



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(Figure 1)

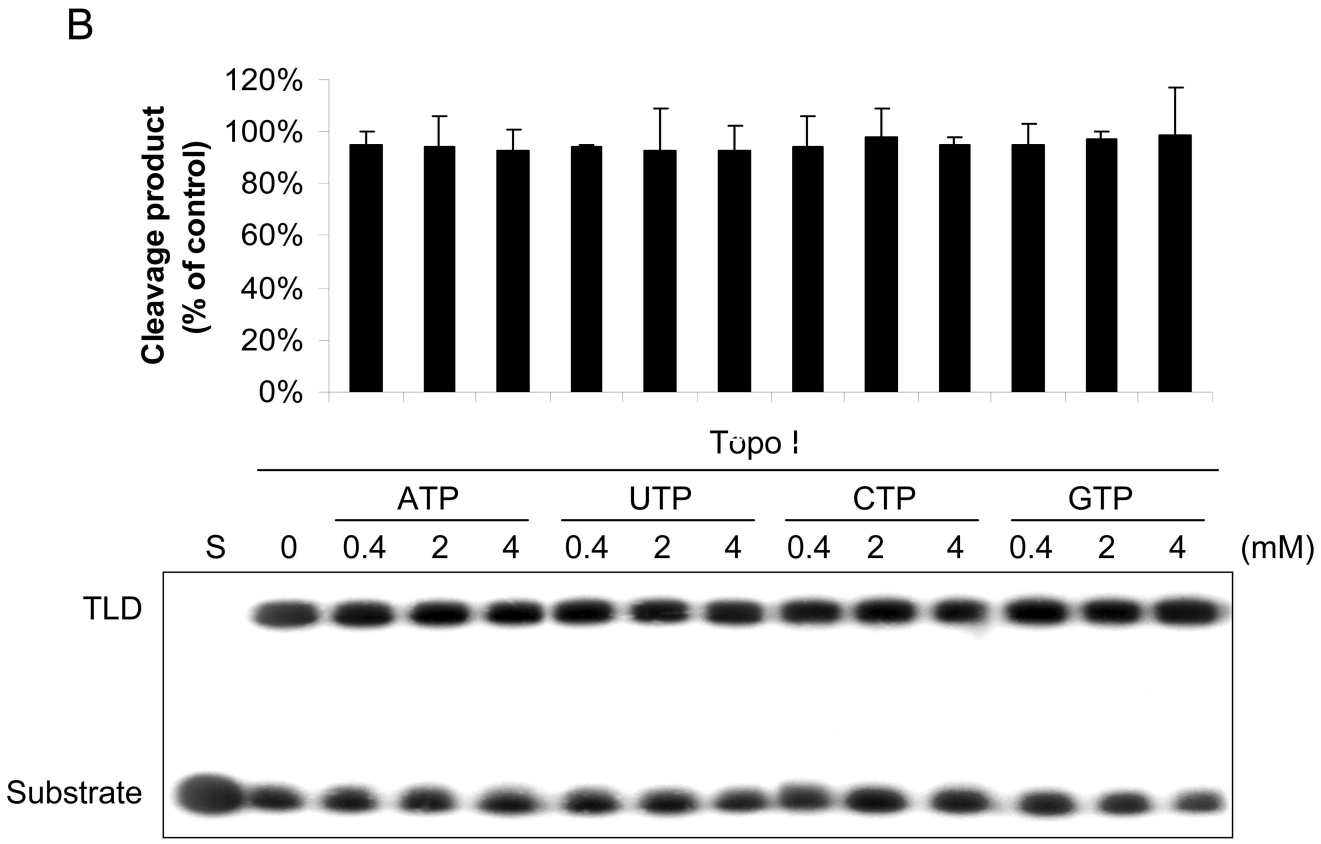

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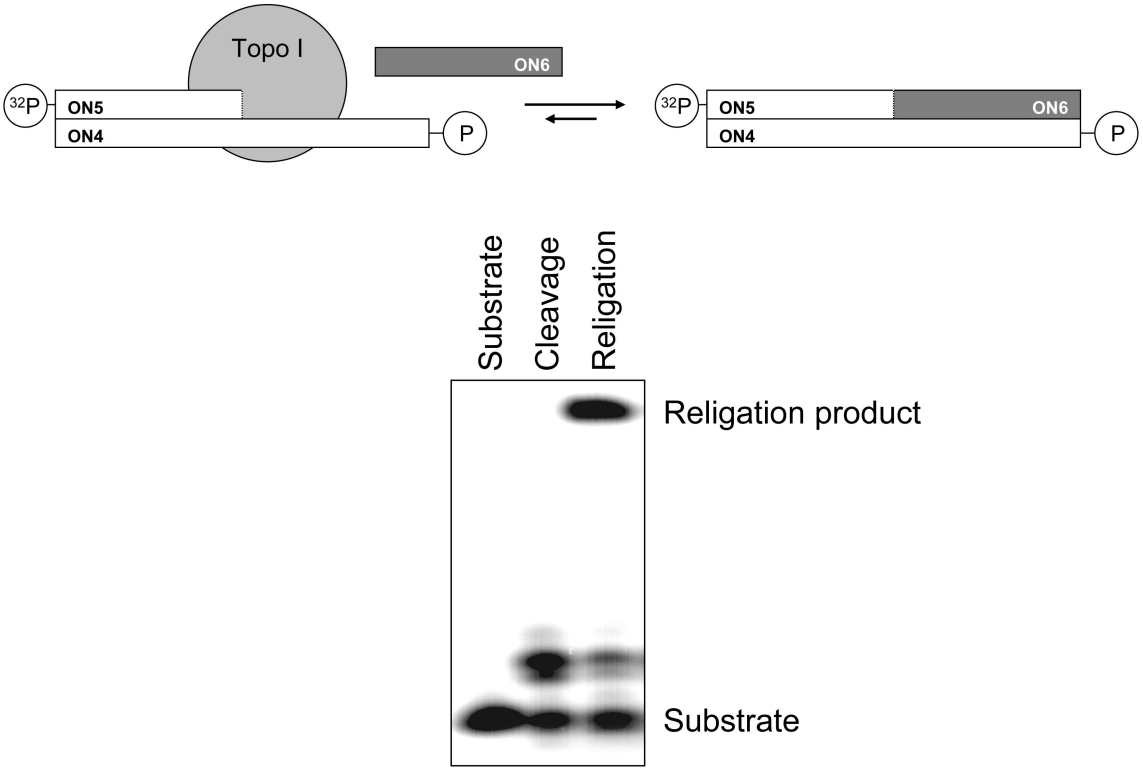
Figure 2

A



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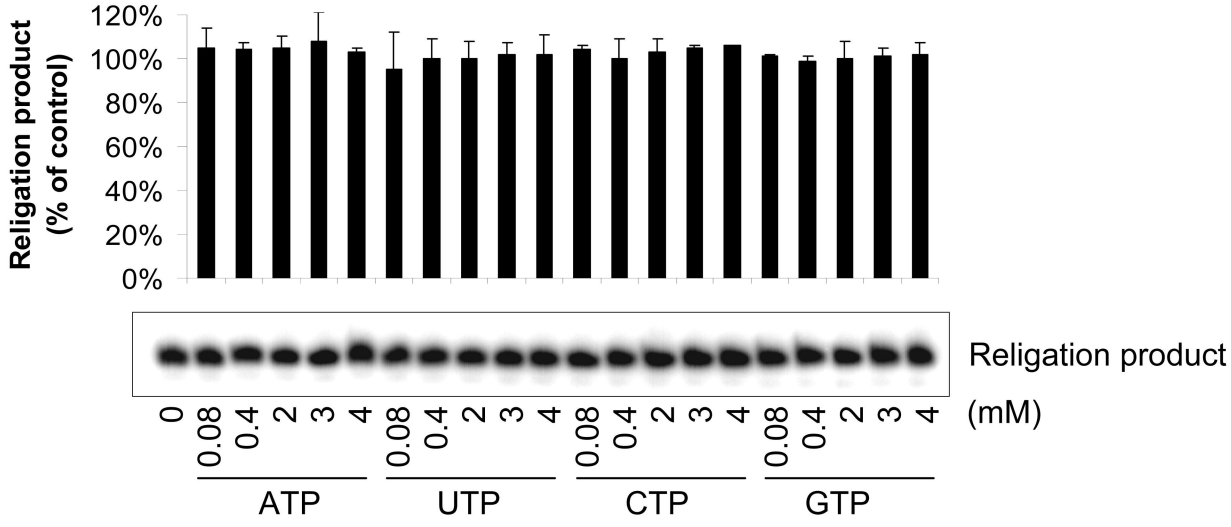

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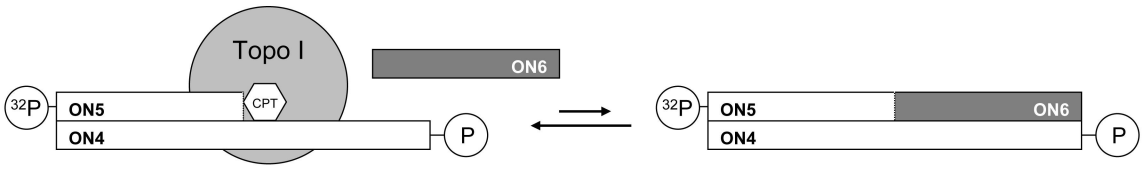
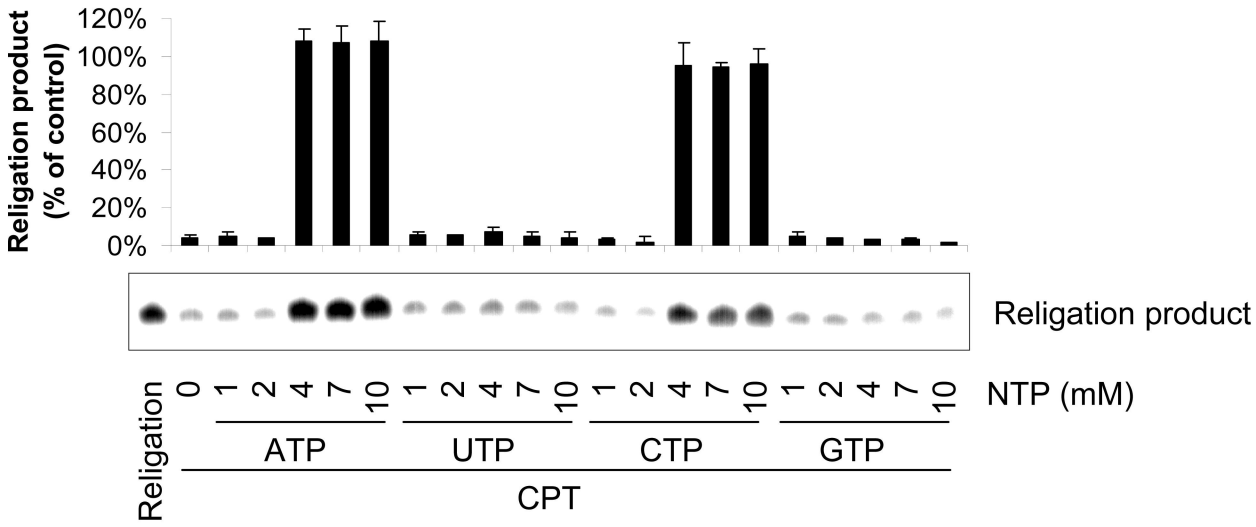
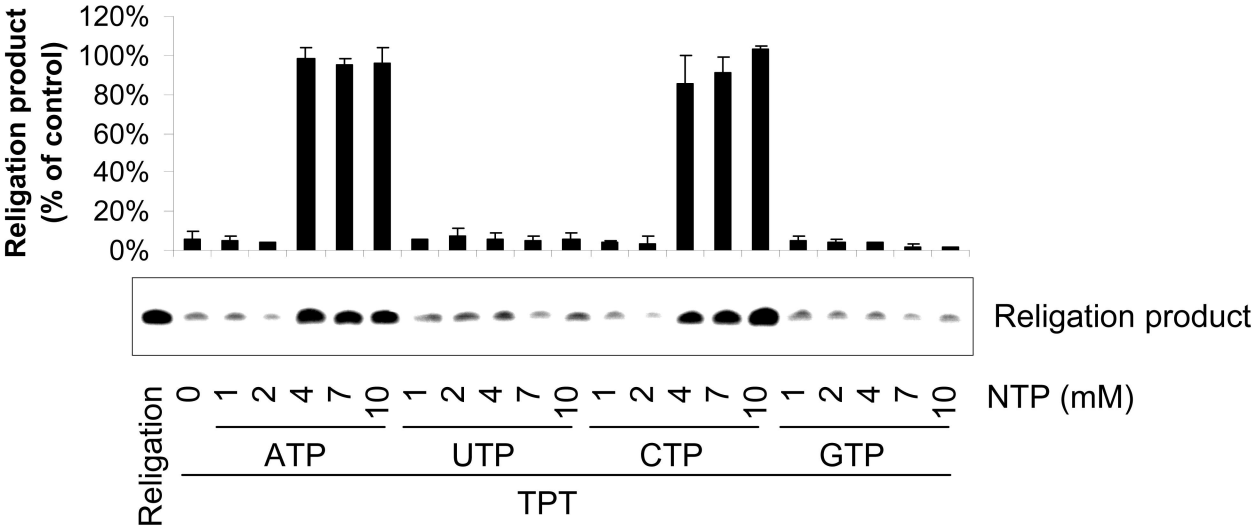
(Figure 2)

C







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(Figure 2)

D

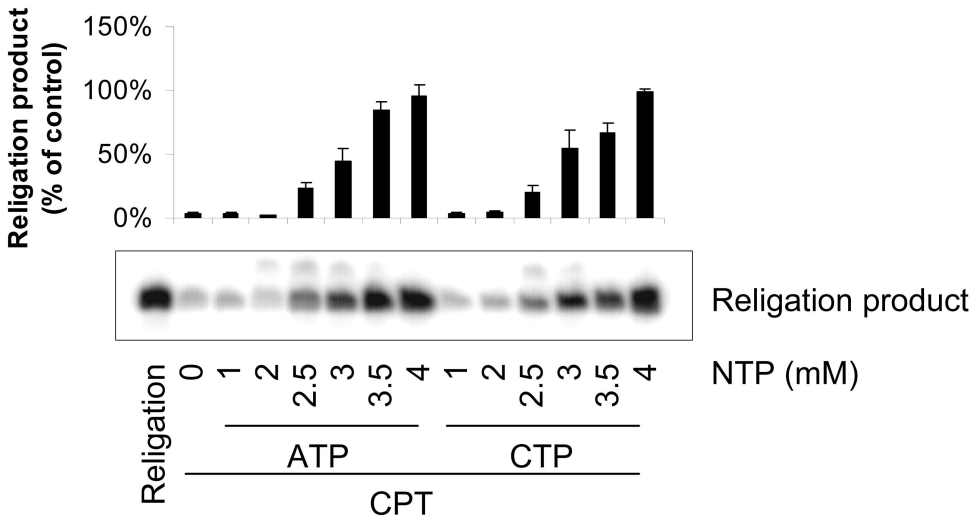

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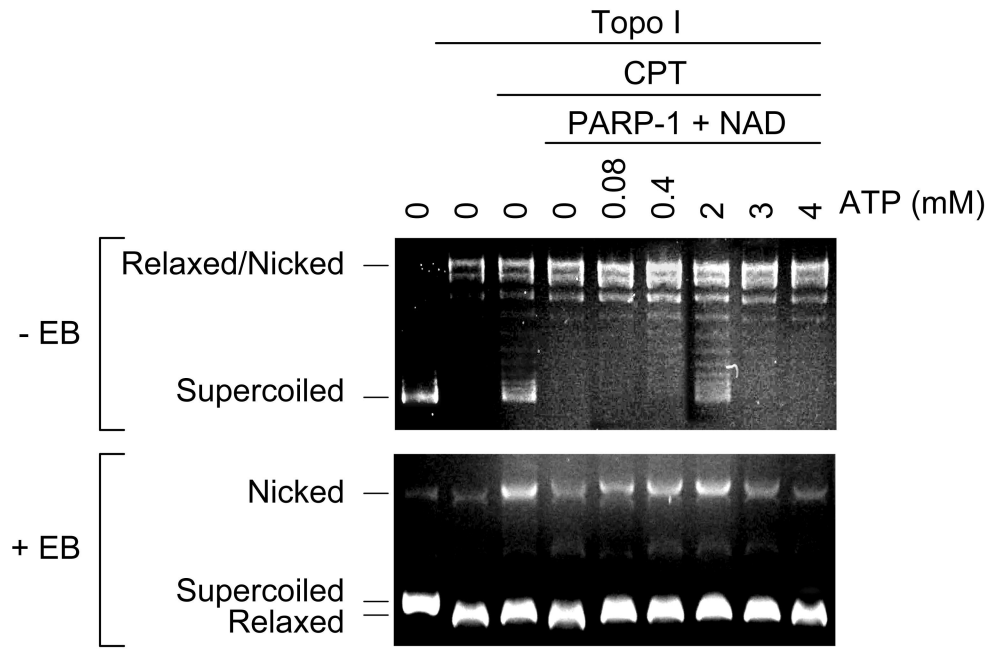
Figure 3

A



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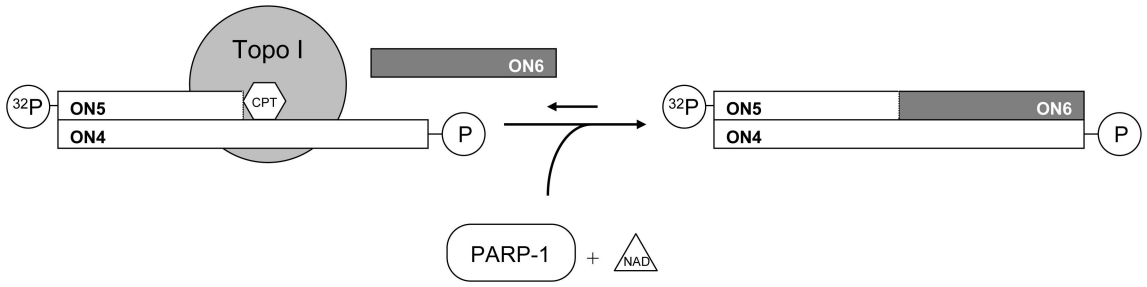
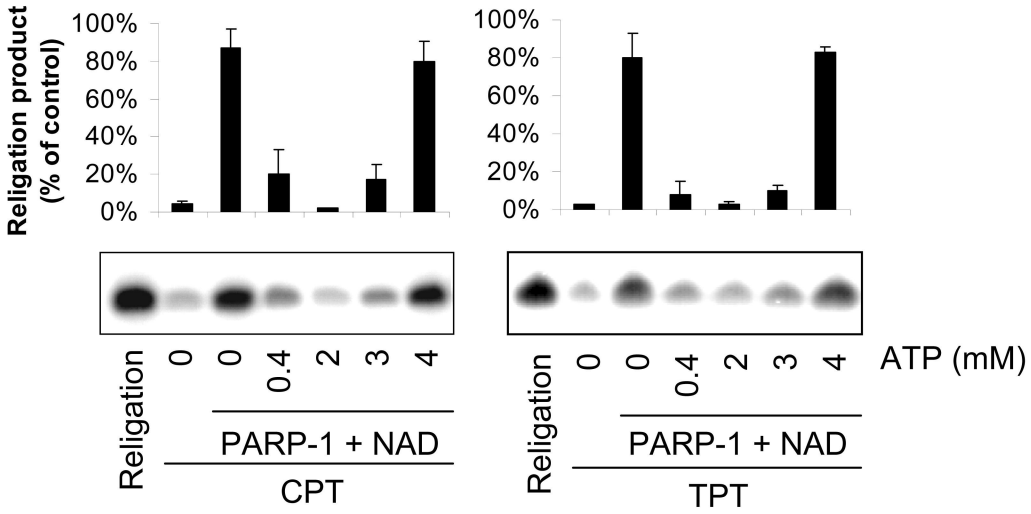
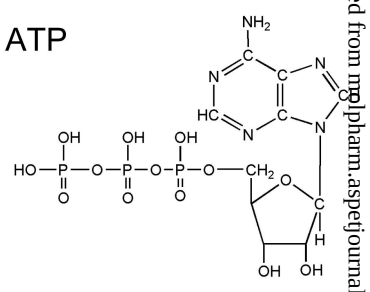
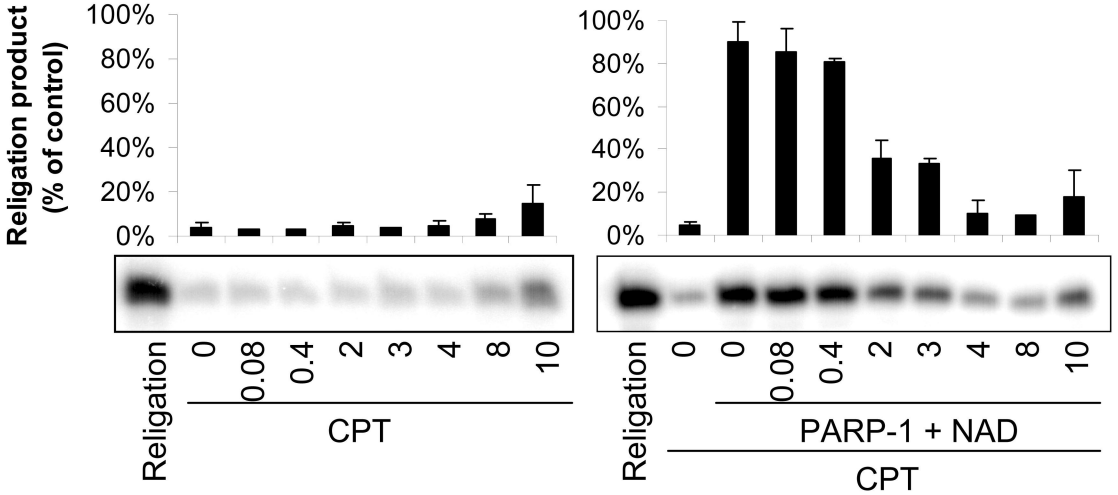
(Figure 3)

B









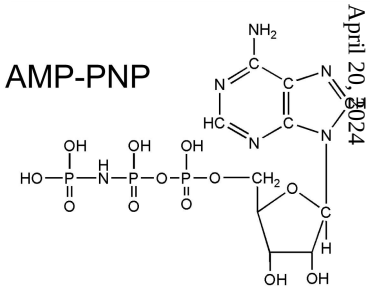


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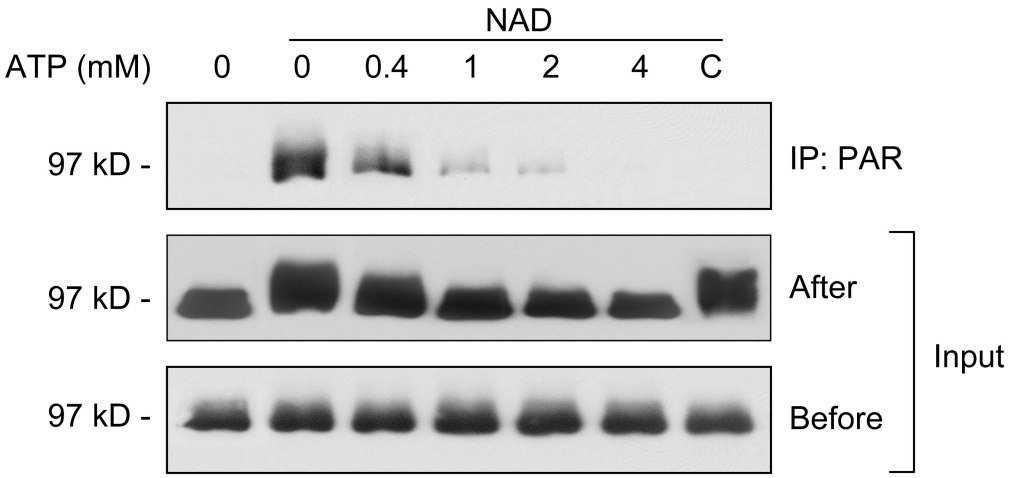
Figure 4

A



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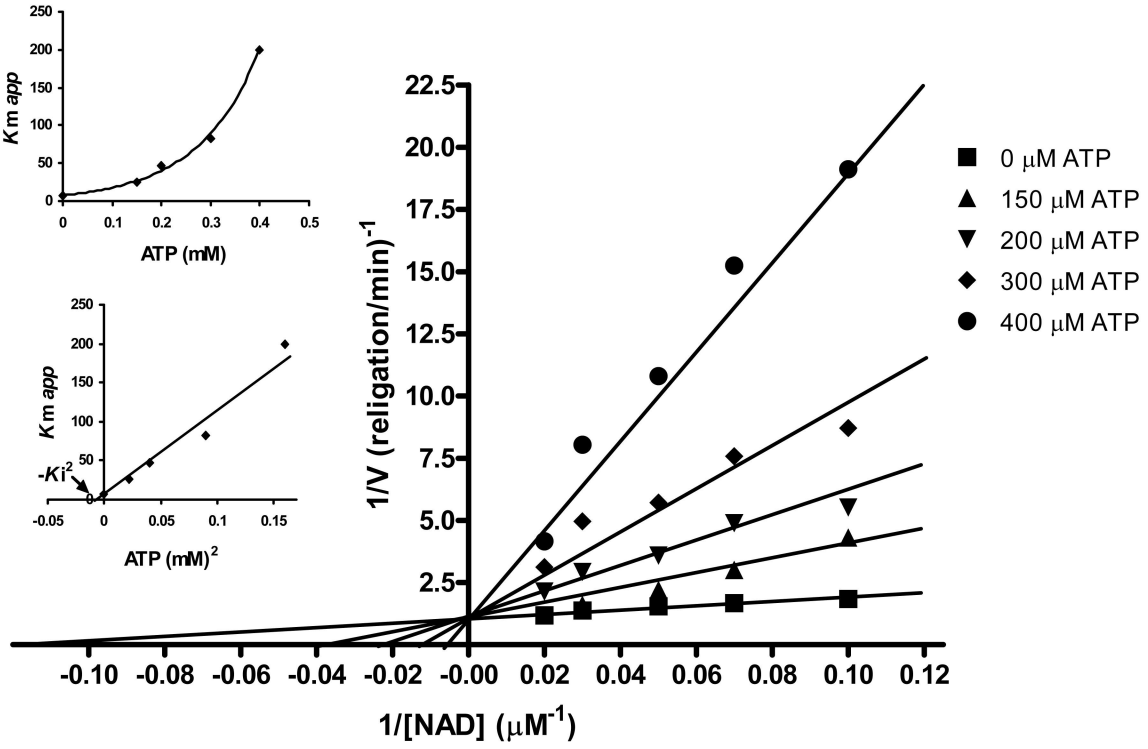

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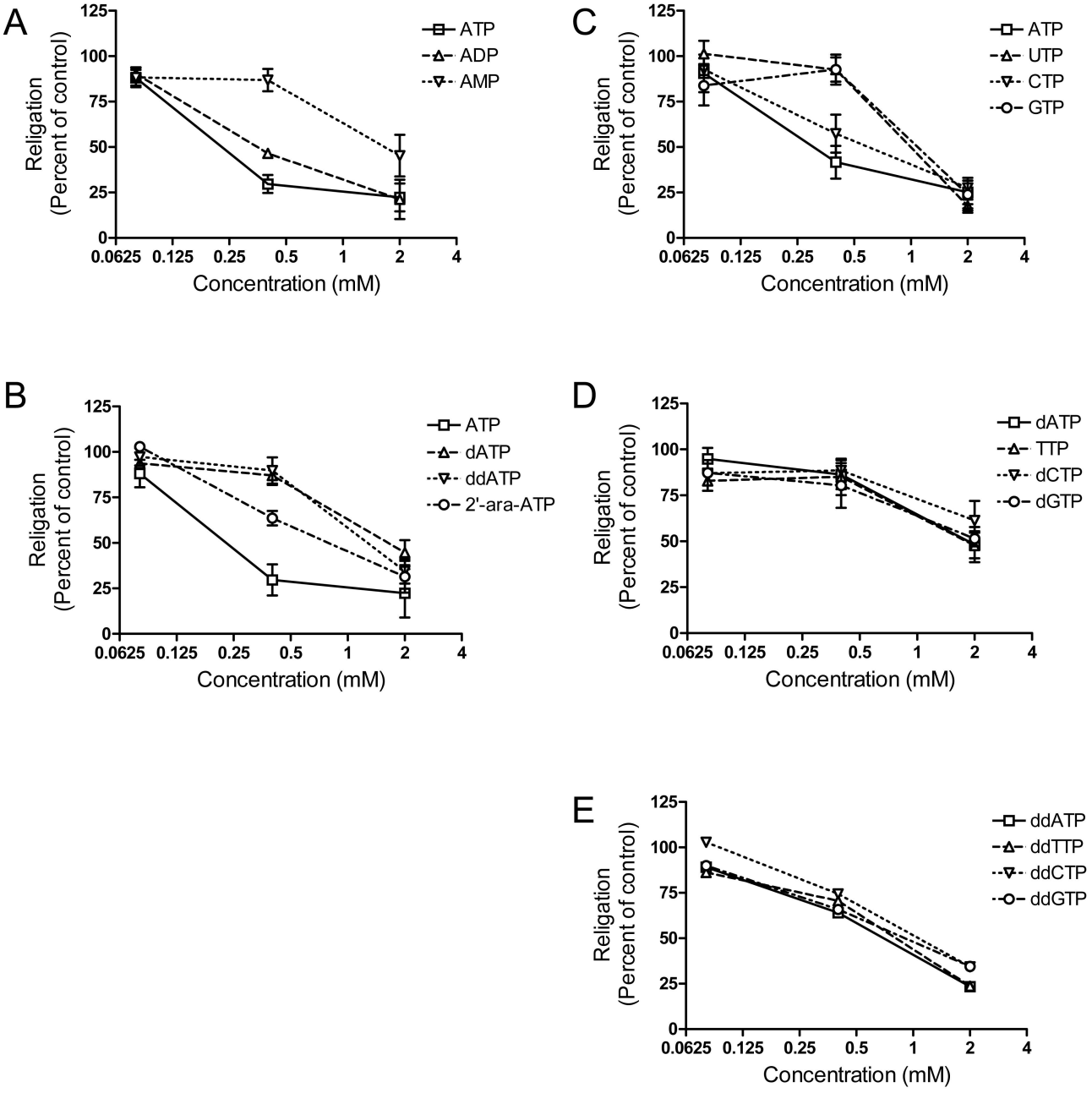
Figure 5



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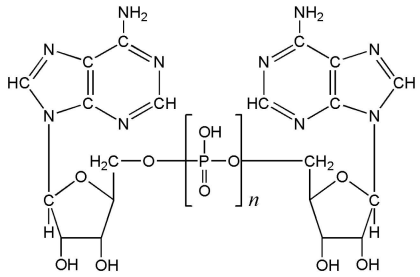
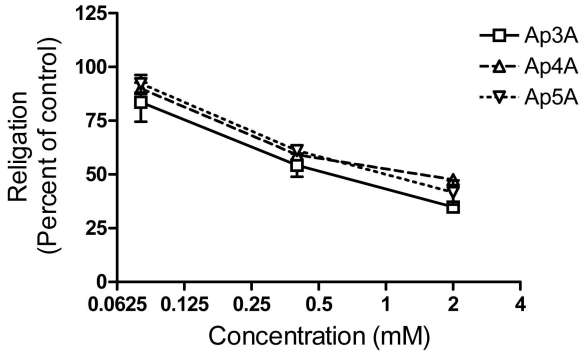
(Figure 5)

F

A[p]*n*A





G

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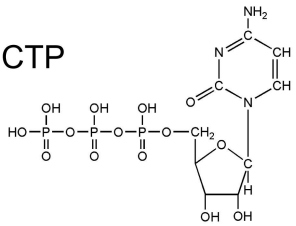

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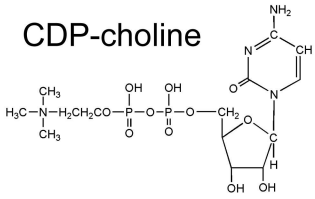

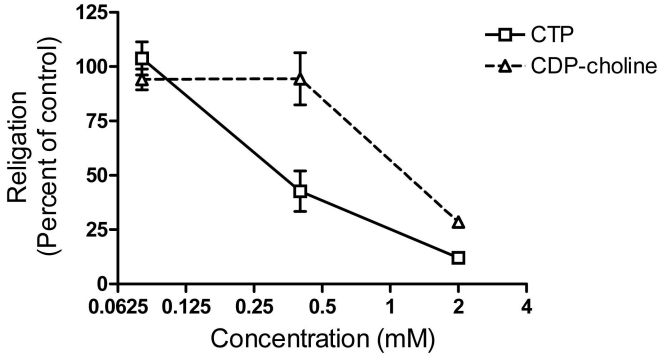

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