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Inhibition of topoisomerase II by 8-chloro-adenosine triphosphate induces DNA double-stranded breaks in 8-chloro-adenosine-exposed human myelocytic leukemia K562 cells

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ABSTRACT

8-Chloro-cAMP and 8-chloro-adenosine (8-Cl-Ado) are known to inhibit proliferation of cancer cells by converting 8-Cl-Ado into an ATP analog, 8-chloro-ATP (8-Cl-ATP). Because type II topoisomerases (Topo II) are ATP-dependent, we infer that 8-Cl-Ado exposure might interfere with Topo II activities and DNA metabolism in cells. We found that 8-Cl-Ado exposure inhibited Topo II-catalytic activities in K562 cells, as revealed by decreased relaxation of the supercoiled pUC19 DNA and inhibited decatenation of the kinetoplast DNA (kDNA). *In vitro* assays showed that 8-Cl-ATP, but not 8-Cl-Ado, could directly inhibit Topo IIα-catalyzed relaxation and decatenation of substrate DNA. Furthermore, 8-Cl-ATP inhibited Topo II-catalyzed ATP hydrolysis and increased salt-stabilized closed clamp. In addition, 8-Cl-Ado exposure decreased bromo-deoxyuridine (BrdU) incorporation into DNA and led to enhanced DNA double-stranded breaks (DSBs) and to increased formation of γ-H2AX nuclear foci in exposed K562 cells. Together, 8-Cl-Ado/8-Cl-ATP can inhibit Topo II activities in cells, thereby inhibiting DNA synthesis and inducing DNA DSBs, which may contribute to 8-Cl-Ado-inhibited proliferation of cancers.

1. Introduction

Topoisomerases resolve topological problems in DNA replication, transcription, recombination, and chromosome condensation/segregation by means of DNA strand breakage and religation [1]. Type I topoisomerases (Topo I) cleave just one strand of DNA, whereas type II topoisomerases (Topo II), which are divided into the IIα and IIβ subclasses [2], cleave both strands.

Eukaryotic type II topoisomerases are homodimeric enzymes with an N-terminal domain capable of binding/hydrolyzing ATP, a central domain critical for DNA breakage/

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Abbreviations: Topo II, topoisomerase II; 8-Cl-Ado, 8-chloro-adenosine; 8-Cl-ATP, 8-chloro-adenosine triphosphate; AMPPNP, adenosine 5′-(β,γ-imido)triphosphate; DSBs, double-stranded breaks; PFGE, pulsed field gel electrophoresis.

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rejoining, and a C-terminal domain important for regulating the enzymes [3]. The reaction cycle for type II enzymes uses a ''two-gate'' mechanism for strand passage [4,5]. The reaction begins with the enzyme binding of one DNA double helix (the G-segment). ATP binding to the N-terminal domains of the subunits brings the two domains together (dimerization), leading to transient DNA double-stranded break (DSB) in the G-segment and to capture of a second double helix (the T-segment). The T-segment then passes through the break in the G-segment and ultimately exits the protein clamp through a C-terminal protein ''gate''. Following religation of the strand break, ATP hydrolysis leads to the dissociation of the N-terminal dimerization, allowing release of the G-segment or initiation of another catalytic cycle. By this way, type II enzymes catalyze DNA catenation/decatenation, knotting/unknotting, and introduction/removal of DNA superturns, altering the linking number of supercoiled DNA [6].

The overall process is orchestrated by the opening and closing of molecular ''gates'' in the enzyme, which is regulated by ATP binding, hydrolysis, and release of ADP. Two ATPs are bound and hydrolyzed during a reaction cycle. Binding of ATP to Topo II is important to N-terminal dimerization [7]. Hydrolysis of two ATP molecules in the reaction cycle occurs sequentially rather than simultaneously [8]. The hydrolysis of one ATP precedes and accelerates the T-segment passage, the second hydrolysis is linked to the resetting of the enzyme [9] through facilitating interdomain communication between the N-terminal ATP binding domain and the central cleavage/ligation domain [10]. Thus, ATP hydrolysis is required for enzyme turnover and rapid kinetics [11]. Although Topo II can relax negatively supercoiled DNA in the absence of ATP, the reaction is very slow [12]. Similarly, in the presence of nonhydrolyzable ATP analog, AMPPNP (adenosine 5′-β,γ-imido-triphosphate), most of the Topo II enzyme is converted to a state in which DNA binding and release is extremely slow, but which allows DNA cleavage [13].

Formation of the covalent enzyme–DNA complex (cleavable complex) is essential for DNA cleavage/religation [1,3]. Classic Topo II inhibitors can trap the cleavable complex, directly poisoning the enzyme and generating DNA DSBs that lead to chromosome damage and cell death [12,14–17]. A diverse group of drugs, so-called ''true'' catalytic inhibitors, inhibit catalytic activity but do not stabilize cleavable complex [15,17–19]. Although the catalytic inhibitors cannot break DNA directly, they are able to increase the stability of the closed clamp form of the enzyme, which is regarded as an obstacle for the progression of the replication fork [20]. Replication fork arrest can be processed to DSBs and recombination [21,22].

8-Chloro-cAMP (8-Cl-cAMP) is an anti-cancer agent that exerts its cytotoxicity by converting into 8-chloro-adenosine (8-Cl-Ado) [23–25]. Cell-cycle arrest and apoptosis are considered to be responsible for this effect. Metabolite analysis demonstrates that 8-Cl-Ado can be phosphorylated to 8-Cl-ATP in cells [25]. 8-Cl-Ado may induce cell growth inhibition by targeting cellular bioenergy and RNA transcription [25–28]. We have previously reported that 8-Cl-Ado can induce G2/M arrest in human lung cancer cells A549 and H1299 [29,30], followed by chromosome segregation aberration [29]. More recently, a study revealed that administration of 8-Cl-cAMP leads to chromosome breaks in mice [31]. We suppose that chromosome segregation aberration and chromosome breaks might be attributed at least in part to the inhibition of Topo II. Because type II enzymes are ATP-dependent and ATP hydrolysis is required for enzyme turnover and rapid kinetics [9–11], we infer that 8-Cl-Ado-converted 8-Cl-ATP, as an ATP analog, might inhibit Topo II activities in cells, thereby leading to incompetent sister chromatid segregation and chromosomal DNA breaks. In this study, we demonstrate that 8-Cl-Ado can inhibit Topo II activities and induce DNA damage in human chronic myelocytic leukemia K562 cells, which might be correlated to 8-Cl-Ado-induced cell-cycle arrest and cell death.

2. Materials and methods

2.1. Cell culture and chemical treatment

Human chronic myelocytic leukemia K562 cell (ATCC, Rockville, MD) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin, and grown in a 37 °C incubator with 5% CO_2.

8-Cl-Ado (The State Laboratory for Natural and Biomimetic Drugs, Peking University HSC, China) was dissolved in sterilized 0.85% NaCl solution and added to cultures at the desired concentration.

2.2. Nuclear extract preparation

Nuclear extracts (NE) were prepared as described [32]. Cells were incubated in 1 ml cold PBS containing 10 mM $MgCl_2$ and 0.35% (v/v) Triton-X-100. After incubation on ice for 10 min, nuclei were pelleted by centrifugation at 1000 × *g* for 10 min. Nuclear proteins were extracted by extraction buffer (20 mM Tris–HCl, pH 7.5, 0.35 M NaCl, 140 mM β-mercaptoethanol, and 50 μg/ml BSA) (Sigma–Aldrich, St. Louis, MO). After incubation on ice for 30 min, lysate was centrifuged at 12,000 × *g* for 5 min. Protein concentration was determined by BCA protein assay kit (Pierce Biotech. Inc., Rockford, IL).

2.3. DNA relaxation

DNA relaxation assay [33] was performed by incubating Topo IIα (TopoGEN, Inc., Columbus, OH) or NE with 400 ng supercoiled pUC19 DNA (MBI Fermentas, Vilnius, Lithuania) in 20 μl relaxation buffer (50 mM Tris–HCl, pH 8, 140 mM KCl, 1 mM EDTA, 8 mM $MgCl_2$ and 2 mM ATP (MBI Fermentas, Vilnius, Lithuania). Reactions were incubated at 37 °C for 30 min and stopped by adding SDS and EDTA to a final concentration of 0.1% and 10 mM, respectively. After proteinase K (1 mg/ml; Roche Diagnostics, Mannheim, Germany) digestion, samples were subjected to electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris acetate, pH 8.2, 1 mM EDTA). DNA was stained with 1 μg/ml ethidium bromide and visualized by UV light.

2.4. Decatenation of kDNA

Decatenation assay [34] was performed with 150 ng kinetoplast DNA (kDNA) (TopoGEN Inc. Columbus, OH) in a 20 μl

reaction containing 50 mM Tris–HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5–2 mM ATP with or without 0.5–8 mM 8-Cl-ATP (BIOLOG, Germany), 30 μg/ml bovine serum albumin and 1 U human Topo IIα or nuclear extracts. Reactions were incubated at 37 °C for 30 min and terminated by adding 5 μl stop buffer (5% N-lauroylsarcosine, 0.125% bromphenol blue, and 25% glycerol). Decatenation was determined by the loss of high molecular weight kDNA (which remained at the gel origin).

2.5. Hydrolysis of ATP by topoisomerase II

ATP hydrolysis was determined as described [35]. Reactions containing 180 nM Topo IIα and 30 nM negatively supercoiled pUC19 were carried out in 100 μl of 50 mM Tris–HCl, pH 7.6, 110 mM KCl, 1 mM EDTA, 8 mM $MgCl_2$, 4 mM phosphoenolpyruvate (Sigma), 0.2 mM NADH (Sigma), 10 μl of pyruvate kinase/lactate dehydrogenase (Sigma), 1 mM ATP and/or 1 mM AMPPNP (Sigma), 1–4 mM 8-Cl-ATP. Mixtures were incubated at 37 °C for 10 min and monitored spectrophotometrically for 10 min at 37 °C. The rate of ATP hydrolysis was monitored as the rate of NADH oxidation and determined by the absorbance decrease at 340 nm.

2.6. Clamp-closing assay

For clamp-closing experiments [35], 2 U Topo IIα was preincubated with 500 ng supercoiled pUC19 DNA for 5 min in 20 μl buffer (50 mM Tris–HCl, pH 8, 140 mM KCl, 1 mM EDTA, 8 mM $MgCl_2$, 1 mM ATP). After preincubation, 1 mM AMPPNP or 1 mM 8-Cl-ATP was added. Reactions were incubated further for 5 min and stopped by adding NaCl to 800 mM. Sample volume was increased to 80 μl by adding 60 μl of 800 mM NaCl solution. To trap enzyme–DNA catenanes, phenol extraction was performed by adding 1 vol. of phenol. Samples were vortexed and centrifuged at 12,000 × *g* for 10 min in Eppendorf tubes. The water phase was removed, ethanol-precipitated, and dissolved in 10 μl TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA) for gel analysis. The combined phenol phase and phenol/water interphase were washed once in high salt washing buffer (2 M NaCl, 50 mM Tris–HCl, pH 7.9, 5 mM $MgCl_2$) and once in 0.6 M LiCl. Upon the removal of the waterphase after the last wash, the remains were ethanol-precipitated and dissolved in 10 μl TE buffer containing 1 mg/ml proteinase K. The samples were subjected to electrophoresis in a 1% agarose gel in TAE (40 mM Tris acetate, pH 8.2, 1 mM EDTA). Gels were stained with 1 μg/ml ethidium bromide and photographed. The supercoiled (SC) and dimeric (D) DNA in the interphase (i) and water (w) phase were estimated by scanning densitometry and analyzed with an Image Master VDS Software (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.7. BrdU incorporation assay

BrdU incorporation was performed using the FITC BrdU Flow Kit (BD Pharmingen, San Diego, CA), according to the manufacturer's instructions. After exposed to 10 μM 8-Cl-Ado, cells were pulsed with 10 μM BrdU for 30 min at 37 °C prior to harvest. Cells were washed in cold staining buffer (1× Dulbecco's phosphate-buffered saline + 3% FBS), fixed/permeabilized with Cytofix/Cytoperm buffer and washed with Perm/Wash buffer (carried out on ice). After permeabilization, cells were treated with 30 μg DNase for 1 h at 37 °C, and stained with FITC-conjugated anti-BrdU antibody and 7-AAD. DNA contents were analyzed by a FACS Canto flow cytometer with FACS Diva software (BD Biosciences, San Jose, CA).

2.8. Pulsed field gel electrophoresis (PFGE)

As described [36], approximately 1.0×10^7 cells were suspended in PBS. Equal volume of 1% agarose prepared in PBS was added at 50 °C. The mixture was immediately poured into molds. Cells embedded in agarose were digested in 5 vol. of lysis buffer (0.5 M EDTA, pH 9, 1% sarcosyl and 0.5 mg/ml proteinase K) for 48 h at 50 °C. DNA plugs were incubated for 1 h in TE buffer containing 0.2 mg/ml RNase A. Agarose plugs containing DNA were inserted into the well of a 0.5% agarose gel. Electrophoresis was carried out using CHEF Mapper XA Pulsed Field Electrophoresis System (Bio-Rad, Munich, Germany). Gels were run for 21 h at 5.6 V/cm with 30–180 s switch time. After electrophoresis, gels were photographed on a UV-transilluminator.

2.9. Immunocytochemistry

As described [37], cells were rinsed with PBS, and fixed in 4% paraformaldehyde in PBS at 4 °C for 15 min, then resuspended in 70% ethanol for 2 h at −20 °C. Cells were washed twice in PBS and suspended in 0.2% Triton-X-100 in 1% BSA solution in PBS for 30 min. After centrifugation cell pellet was suspended in 100 μl of 1% BSA containing 1:2000 diluted anti-γ-H2AX antibodies (R&D Systems, Inc., Minneapolis, MO) and incubated overnight at 4 °C. After washing, cells were incubated with 100 μl of 1:100 diluted Rhodamine-conjugated anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature in the dark. After washing, nuclei were stained with 5 μg/ml Hoechst 33342 (Molecular Probes) for 20 min at room temperature. The cells were dropped on slides and viewed with Leica TCS SP2 confocal microscopy (Leica Microsystems Heidelberg GmbH, Mannheim, Germany).

2.10. Western blotting

Cells were lysed, and proteins in lysates were quantified. Fifty micrograms of proteins were subjected to SDS-PAGE and transferred onto nitrocellulose membranes, blocked with 5% nonfat milk in TBS-T (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20) at room temperature for 2 h with rocking. The membranes were probed with anti-γ-H2AX antibodies (R&D Systems, Inc.) overnight at 4 °C. After washing with 5% nonfat milk/TBS-T, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in 5% nonfat milk/TBS-T at room temperature for 1 h. The protein–antibody complex was detected by Western blotting luminol reagent (Santa Cruz Biotechnology Inc.)

3. Results

3.1. Topo II activities are decreased in 8-Cl-Ado-exposed K562 cells

K562 cells were exposed to 0–20 μM 8-Cl-Ado for 48 h, and nuclear extracts were prepared. The topoisomerases activities in the NE were examined by the relaxation of supercoiled pUC19 DNA. As shown in Fig. 1A, the supercoiled DNA was relaxed, when the substrate DNA was incubated with the NE from unexposed K562 (lane 2), indicating the constitutive activities of topoisomerases in the nuclei. However, the relaxation was inhibited in an 8-Cl-Ado concentration-dependent fashion from 2 to 20 μM (lanes 3–6), when the substrate was incubated with the NE from exposed cells. These results indicate that 8-Cl-Ado exposure can decrease the topoisomerases activities in exposed cells.

Type II enzymes are ATP-dependent [9–11], we therefore infer that 8-Cl-Ado-converted 8-Cl-ATP, as an ATP analog, might inhibit Topo II activities in cells. To test this idea, DNA decatenation assay was performed using the kinetoplast DNA which is a specific substrate for the Topo II enzymes [38]. A typical result of decatenation reaction is shown in Fig. 1B. When kDNA was incubated with the NE from unexposed K562 cells, almost all catenated circles of substrate kDNA were decatenated as minicircular monomeric DNA (Fig. 1B, lane 3). However, when the substrate DNA was incubated with the NE from 8-Cl-Ado-exposed K562, the decatenation reactions were inhibited in varying degrees (lanes 4–10). The decatenation activity was markedly inhibited in the NE from the cells exposed to 5–20 μM 8-Cl-Ado (lanes 7–9) and almost completely abolished in the NE from 50 μM 8-Cl-Ado-exposed cells (lane 10). These results indicate that 8-Cl-Ado exposure can lead to decrease of Topo II activities in exposed cells.

3.2. 8-Cl-ATP but not 8-Cl-Ado competes with ATP to inhibit Topo II-dependent relaxation

8-Cl-Ado undergoes phosphorylation conversion into 8-Cl-ATP in cells [25]. To differentiate any potential inhibitory role of 8-Cl-ATP from that of 8-Cl-Ado in exposed cells, we examined the relaxation of supercoiled pUC19 with purified Topo IIα in the presence of 8-Cl-ATP or 8-Cl-Ado. Compared with the relaxation in the presence of 2 mM ATP (Fig. 2A, lane 2), 0.5–8.0 mM 8-Cl-Ado exhibited undetectable inhibitory effects on the relaxations by Topo IIα (Fig. 2A, lanes 3–8), whereas 1.5–8.0 mM 8-Cl-ATP markedly inhibited the relaxations (Fig. 2B, lanes 5–8). These results indicate that 8-Cl-ATP directly inhibits the relaxation activity of Topo II and may compete with ATP for the enzyme, while 8-Cl-Ado has insignificant effect. Unsurprisingly, in the presence of 8-Cl-Ado or 8-Cl-ATP but without ATP, Topo IIα could not relax the supercoiled substrates at all (Fig. 2A and B, lane 9), because Topo II is ATP-dependent.

To further demonstrate whether 8-Cl-ATP competes with ATP for the enzyme, we carried out relaxation reaction using varied moles of ATP in the presence of 2 mM 8-Cl-ATP. We found that the increased ATP (>1.5 mM) could prevent 8-Cl-ATP-inhibited relaxation (Fig. 2C). The results from Fig. 2B and C suggest that the inhibition of Topo IIα-catalyzed relaxation by 8-Cl-ATP depends on the ratios between ATP and 8-Cl-ATP.

3.3. 8-Cl-ATP inhibits Topo II-catalyzed ATP hydrolysis

To further study the inhibitory mechanism of Topo II enzymes by 8-Cl-ATP, we tested the effects of 8-Cl-ATP on Topo II-catalyzed ATP hydrolysis by examining the rate of ATP hydrolysis. In the reactions containing 180 nM Topo IIα, 1 mM ATP and 30 nM negative supercoiled pUC19 DNA (Fig. 3), Topo IIα displayed almost 50% decreases in ATP hydrolysis activity in the presence of 1 mM 8-Cl-ATP,

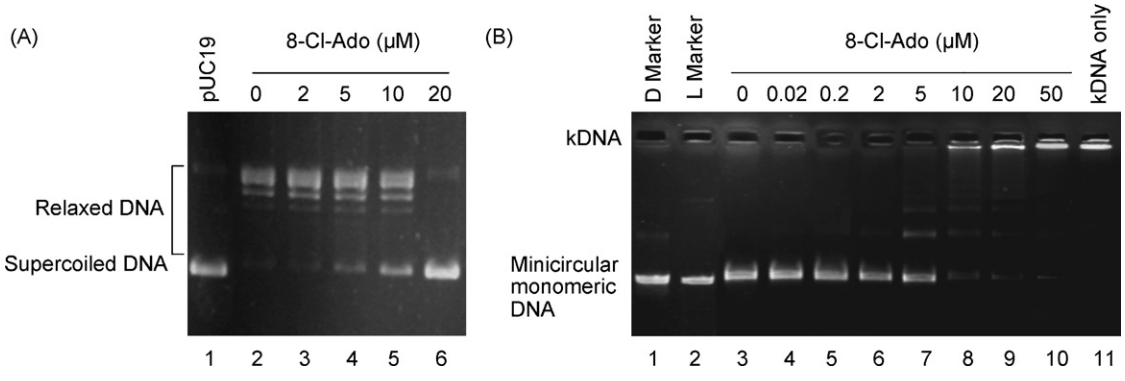

Fig. 1 – 8-Cl-Ado exposure specifically inhibits type II topoisomerase activities in K562 cells. (A) Inhibition of topoisomerase-catalyzed relaxation by 8-Cl-Ado exposure. K562 cells were exposed to 8-Cl-Ado at the indicated concentrations for 48 h, and the nuclear extracts (NE) were prepared. The relaxation activities in NE were tested by incubating with supercoiled pUC19 DNA. After proteinase K digestion, the samples were subjected to 1% agarose gel electrophoresis. The pUC19 DNA without NE in lane 1 is used as markers for supercoiled and relaxed DNA. (B) Inhibition of Topo II activities in 8-Cl-Ado-exposed NE. K562 cells were exposed to 8-Cl-Ado at the indicated concentrations for 48 h, and the nuclear extracts were prepared. Decatenation assay was performed with 150 ng substrate kinetoplast DNA (kDNA) in a 20 μl reaction buffer containing 2 mM ATP and 2.5 μg NE. The D Marker (lane 1) and L Marker (lane 2) represent decatenated and linear kDNA marker in the kit, respectively.

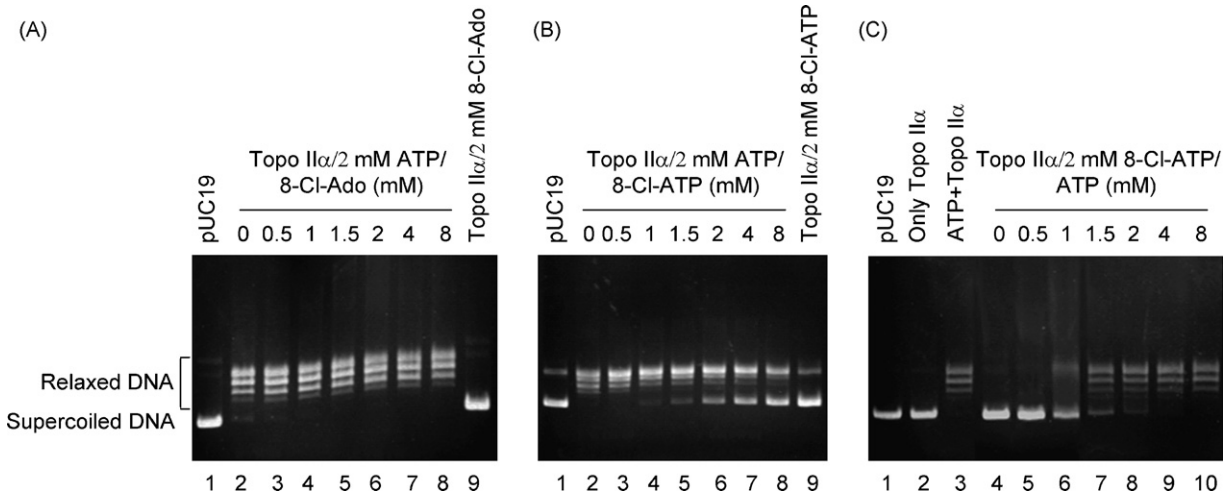

Fig. 2 – 8-Cl-ATP but not 8-Cl-Ado can inhibit topoisomerase II-catalyzed relaxation. Supercoiled pUC19 DNA was incubated with 1 unit of Topo IIα in the presence of 2 mM ATP and of 0–8.0 mM 8-Cl-Ado (A) or 2 mM ATP and 0–8.0 mM 8-Cl-ATP (B) or 2 mM 8-Cl-ATP and 0-8.0 mM ATP (C). After reaction and proteinase K digestion, the samples were subjected to 1% agarose gel electrophoresis. The pUC19 DNA without Topo IIα in lane 1 in (A), (B) and (C) is used as DNA control. Lane 2 in (C) is control of pUC19 with Topo IIα in the absence of ATP. Lane 3 in (C) is control of pUC19 with Topo IIα in the presence of 2 mM ATP.

compared with ATP hydrolysis in the absence of 8-Cl-ATP. This result was similar to that in the presence of AMPPNP, a well-known nonhydrolyzable ATP analog (Fig. 3A). Furthermore, increased concentrations of 8-Cl-ATP continued to reduce the Topo IIα-catalyzed ATP hydrolysis to a rate similar to that of DNA-independent ATP hydrolysis (Fig. 3B). These results indicate that 8-Cl-ATP can inhibit Topo IIα-catalyzed ATP hydrolysis.

3.4. *8-Cl-ATP increases formation of salt-stable closed clamp complex*

To determine the effect of 8-Cl-ATP on the formation of the closed clamp form of Topo II, clamp-closing assays were performed. In these assays, Topo IIα was preincubated with supercoiled pUC19 and ATP before adding 8-Cl-ATP or AMPPNP. The trapped enzyme–DNA catenates were extracted with phenol, followed by washing with high salt buffer. After the samples were subjected to electrophoresis in a 1% agarose gel containing ethidium bromide (Fig. 4A), densitometry scans of the respective bands were obtained using Image Master VDS software (Fig. 4B). As shown in Fig. 4, lanes 3 and 4, Topo II and ATP did not lead to retention of DNA as a salt-stable complex with Topo IIα, which could be collected from the phenol/water interphase (i), while DNA was detected in the water phase (w). In the presence of 8-Cl-ATP, however, the DNA occurred in both the interphase and waterphase. Especially, closed circular DNA was trapped in the interphase (Fig. 4, lanes 5–8) which was similar to that in the presence of AMPPNP (Fig. 4, lanes 9–12), indicating that 8-Cl-ATP can increase the formation of salt-stable enzyme–DNA complex.

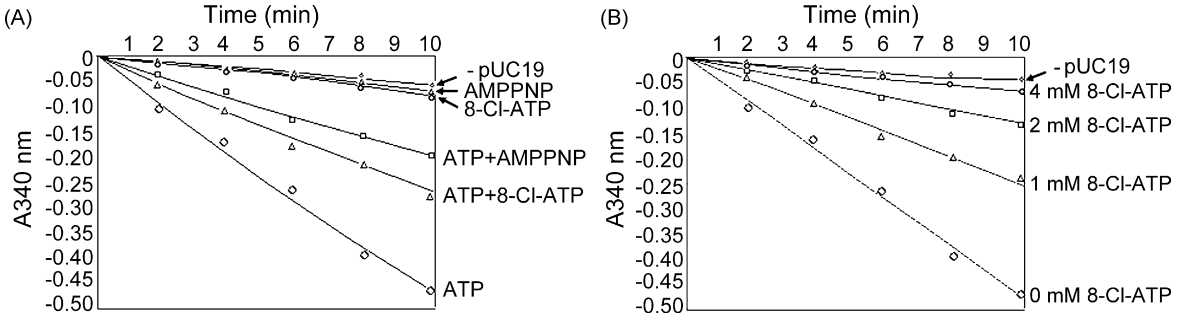

Fig. 3 – 8-Cl-ATP inhibits Topo II-catalyzed ATP hydrolysis. (A) Inhibition of ATP hydrolysis by 8-Cl-ATP or AMPPNP. ATP hydrolysis was performed as described in Section 2. ATP hydrolysis was monitored as the rate of NADH oxidation, which was spectrophotometrically determined by the absorbance decrease at 340 nm. (B) Inhibition of ATP hydrolysis by 8-Cl-ATP in a dose-dependent manner. Analyses of ATP hydrolysis were performed as in Fig. 3A. 1 mM ATP was used as substrate. −pUC19, without supercoiled pUC19 DNA.

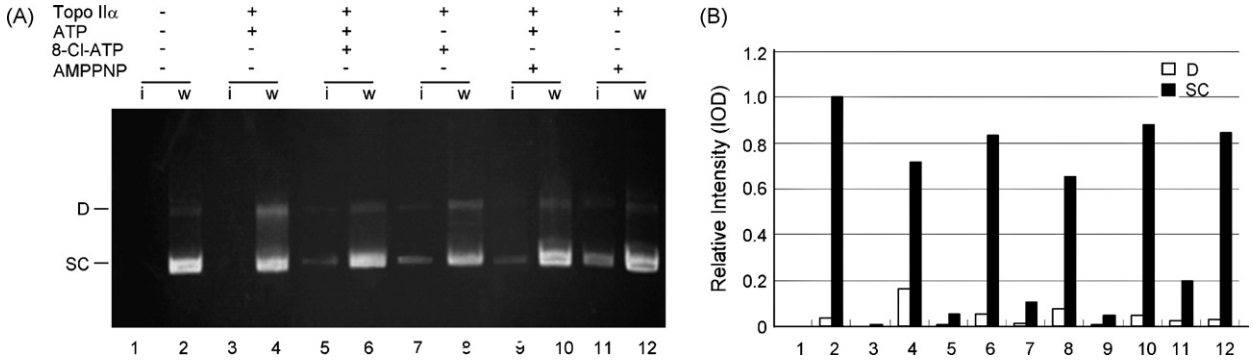

Fig. 4 – 8-Cl-ATP increases salt-stable closed clamp. (A) The formation of salt-stable closed clamp by 8-Cl-ATP. Closed clamp assays were initiated with preincubation of pure Topo IIα (2 U) with supercoiled pUC19 DNA in 20 μl of reaction buffer in the presence or absence of ATP (1 mM). After preincubation, 1 mM AMPPNP or 1 mM 8-Cl-ATP was added to the reaction. The reactions were stopped by adding 800 mM NaCl. The trapped enzyme–DNA catenanes were extracted with phenol, followed by washing in high salt buffer. DNA was collected from the phenol/water interphase (i) and from the water phase (w) and ethanol-precipitated, followed by 1% agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed. The positions of supercoiled (SC) and dimeric (D) plasmid DNA are indicated. (B) Histogram of the density values of supercoiled and dimeric plasmid DNA. Densitometry scanning of the respective bands in Fig. 4A was performed using Image Master VDS software. The density value of the "SC" band in lane 2 is normalized to one. The relative intensity is present as integrated optical density (IOD). The data from one of three independent experiments is shown.

3.5. *8-Cl-ATP inhibits Topo II-specific multiple turnover decatenation*

Next, we examined *in vitro* inhibition of Topo II decatenation activities by 8-Cl-ATP, using the kDNA decatenation assay. As shown in Fig. 5A, the kDNA displayed drastic decatenation by Topo IIα in an ATP-dependent manner. However, such decatenation was partially inhibited when the ATP/8-Cl-ATP molar ratios were higher than 1 (2 mM ATP/0.5–1.5 mM 8-Cl-ATP in Fig. 5B, lanes 4–6), and was completely abolished form in the well, when ATP/8-Cl-ATP ratios were less than 1 (2 mM ATP/2–8 mM 8-Cl-ATP in Fig. 5B, lanes 7–9). These results indicate that 8-Cl-ATP competes efficiently with ATP for the Topo II enzymes, specifically inhibiting Topo II-dependent multiple turnover decatenation via its nonhydrolyzable ability.

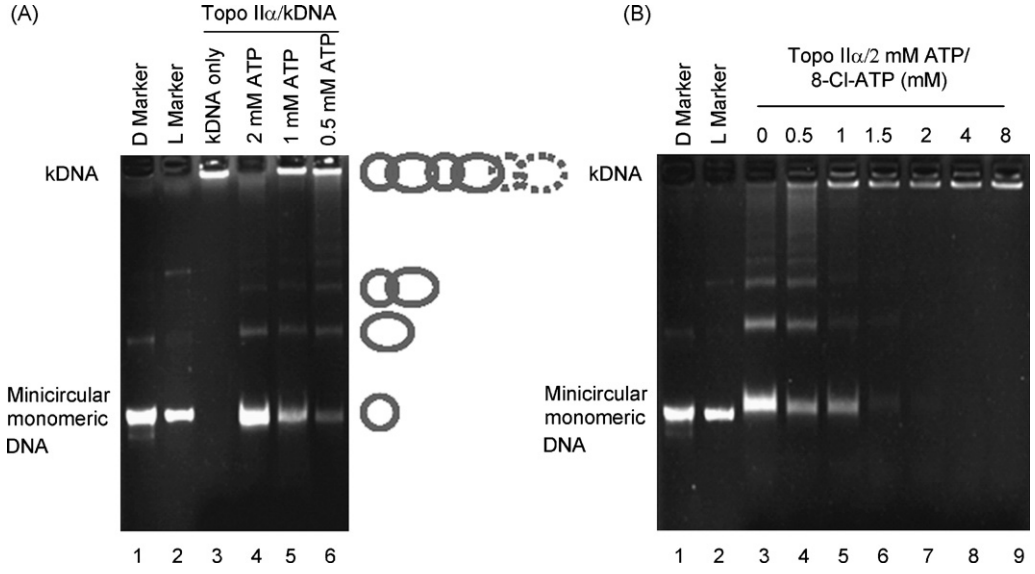

Fig. 5 – 8-Cl-ATP inhibits Topo II-catalyzed multiple turnover decatenation. (A) ATP-dependent multiple decatenation of kDNA by Topo IIα. Decatenation assay was performed with 150 ng substrate kinetoplast DNA (kDNA) in a 20 μl reaction containing 0.5–2.0 mM ATP and 1 unit human Topo IIα. The levels of decatenation were determined by the loss of high molecular weight kDNA (which remained at the gel origin). The D Marker (lane 1) and L Marker (lane 2) represent decatenated and linear kDNA marker in the kit, respectively. The sketch map (right) shows the substrate kDNA and decatenated products. (B) Inhibition of Topo IIα-catalyzed multiple decatenation by 8-Cl-ATP in a dose-dependent manner.

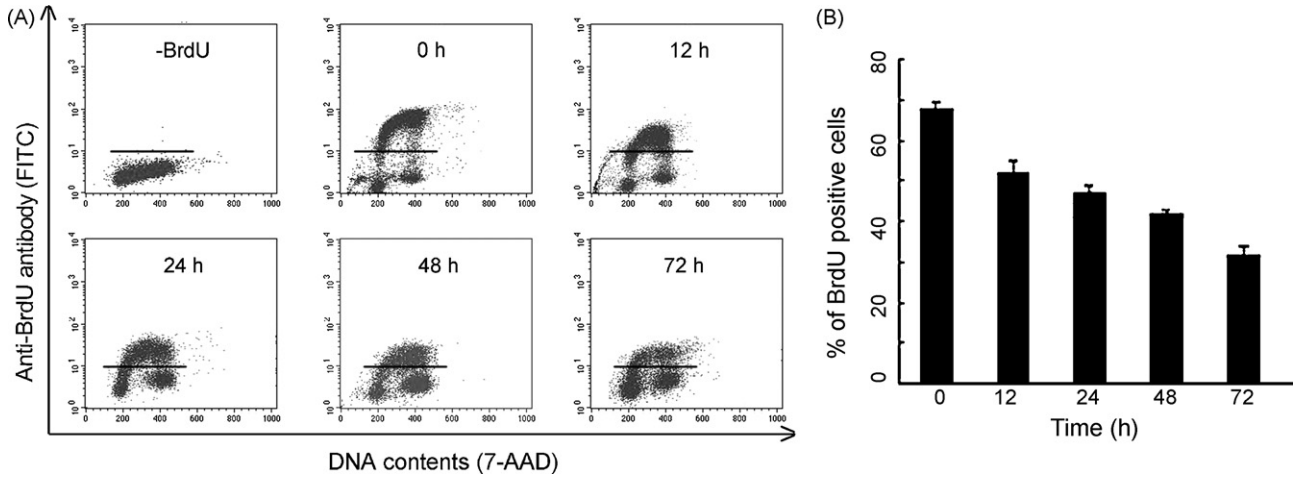

Fig. 6 – 8-Cl-Ado exposure inhibits BrdU incorporation into DNA in K562 cells. Analysis of BrdU incorporation was performed using the FITC BrdU Flow Kit. K562 cells were exposed to 10 μM 8-Cl-Ado for 12–72 h and subsequently pulsed with 10 μM BrdU for 30 min at 37 °C prior to harvest. After fixation/permeabilization, cells were treated with DNase and stained with FITC-conjugated anti-BrdU antibody. DNA contents were analyzed by a FACS Canto flow cytometer with FACS Diva software. (A) Flow cytometric analysis of BrdU-positive cells. The cell untreated with BrdU is used as blank. (B) Histograms showing the percentage of BrdU-positive K562 cells. Data represent mean ± S.D. (n = 3).

3.6. *8-Cl-Ado exposure inhibits BrdU incorporation into DNA in K562 cells*

Because topoisomerases resolve the winding problems arisen from DNA replication, the inhibition of these enzymes may lead to attenuated DNA synthesis. We thus analyzed proliferating cells for their DNA synthesis activities in 8-Cl-Ado-exposed cells. After K562 cells were exposed to 8-Cl-Ado in the presence of bromo-deoxyuridine (BrdU), cells were stained with a fluorescent anti-BrdU antibody and analyzed by flow cytometry (Fig. 6A). The numbers of BrdU-labeled K562 cells were reduced from 51% to 32% in exposed cells within 12–72 h, compared with 68% of BrdU-labeled cells in mock-treated controls (Fig. 6B). The reduction of BrdU-incorporated cells indicates that 8-Cl-Ado exposure causes the inhibition of DNA synthesis, which is likely associated with the inhibition of Topo II.

3.7. *8-Cl-Ado exposure induces DNA double-stranded breaks (DSBs)*

Since 8-Cl-ATP increased the formation of closed clamp (Fig. 4), which can represent an obstacle for the progression of the replication fork [20] and replication fork arrest can be processed to DNA DSBs [21,22], we examined DSBs in 8-Cl-Ado-exposed cells. After exposure of K562 and HL60 cells to 8-Cl-Ado, the genomic DNA was extracted and subjected to pulsed field gel electrophoresis (PFGE) (Fig. 7A). Compared with unexposed DNA, 8-Cl-Ado-treated DNA showed a significant decrease of high molecular weight DNA, accompanied by a marked increase in the release of low molecular weight DNA into the gel. The increased DNA fragmentation strongly suggests that 8-Cl-Ado exposure generates damaged DNA, mostly likely derived from DNA double-stranded breaks.

When cells encounter DSBs, the γ-H2AX (phospho-H2AX-Ser139), a specific marker of DSBs, is immediately generated, forming discrete nuclear foci at the damaged sites [39]. To further demonstrate 8-Cl-Ado-induced DNA DSBs, we examined the formation of γ-H2AX nuclear foci in exposed K562 cells by immunohistochemical labeling. As shown in Fig. 7B, the numbers of γ-H2AX foci were significantly increased within 12–48 h after exposure. In consistence with the increase of γ-H2AX foci, elevated levels of γ-H2AX protein in exposed cells were also revealed by Western blotting (Fig. 7C). These results suggest that 8-Cl-Ado exposure induces DNA DSBs in cells.

4. Discussion

Although 8-Cl-cAMP exerting its cytotoxicity has been known for about 20 years, many features of its molecular mechanism still remain to be fully elucidated. It is generally accepted that there are two major models to explain 8-Cl-cAMP actions. According to the first model, the antiproliferating effect is attributed directly to the pro-drug 8-Cl-cAMP. For example, 8-Cl-cAMP may induce down-regulation of the RIα subunit and up-regulation of the RIIβ subunit of protein kinase A, leading to type II holoenzyme-dependent growth inhibition and differentiation [40]. In the second model, 8-Cl-cAMP and 8-Cl-Ado inhibit proliferation of cancer cells by the same mechanism [23–25], in which the core is conversion of 8-Cl-Ado into 8-Cl-ATP [25]. Both 8-Cl-cAMP and 8-Cl-Ado can activate protein kinase C and down-regulate Cyclin B, inducing growth inhibition [41], or activate p38 MAP kinase-mediated apoptosis [42]. Importantly, 8-Cl-Ado-converted 8-Cl-ATP may target cellular bioenergy [25,26] and inhibit RNA synthesis by chain termination [26,27] or by inhibition of poly(A) polymerase [28]. However, little is known regarding

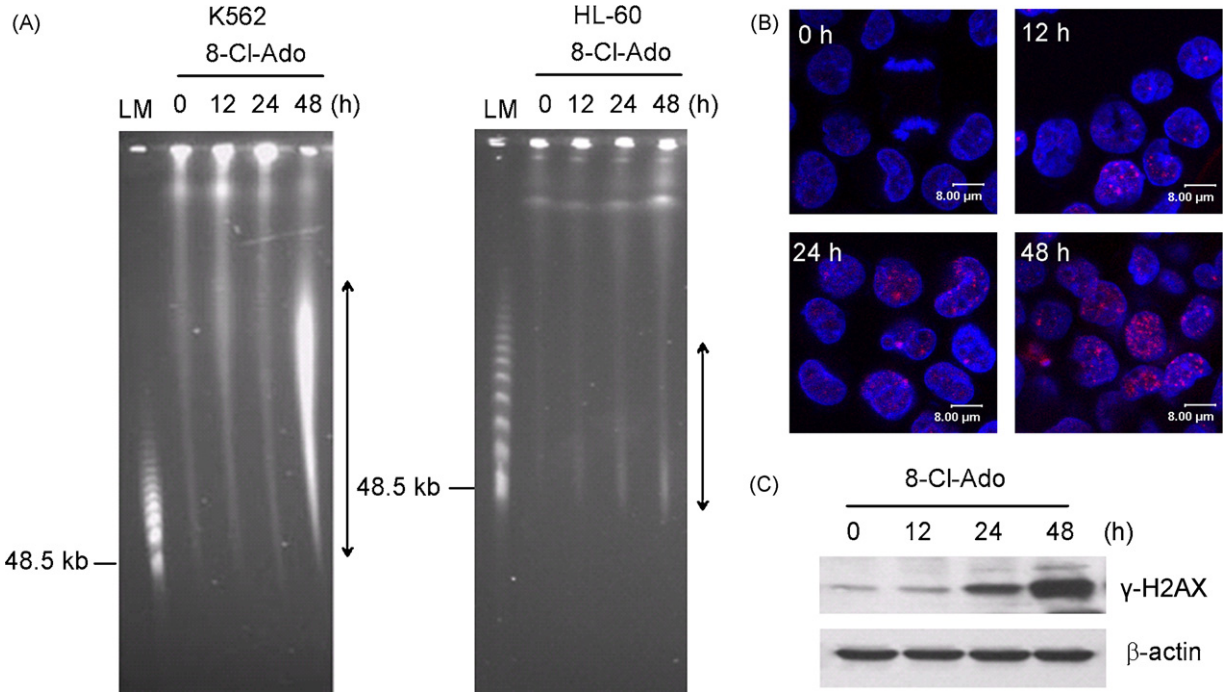

Fig. 7 – 8-Cl-Ado exposure induces DNA double-strand breaks (DSBs). (A) Pulsed field gel electrophoresis (PFGE) analysis of DSBs induced by 8-Cl-Ado expose. K562 and HL60 cells were exposed to 10 μM 8-Cl-Ado for indicated time. After harvested, approximately 1.0×10^7 cells were suspended in PBS and equal volume of 1% agarose at 50 °C in molds. Cells embedded in agarose were digested, and the DNA plugs were prepared as described in Section 2. Agarose plugs contained DNA were inserted into the well of a 0.5% agarose gel. DNA DSB analyses by PFGE were carried out, using CHEF Mapper XA Pulsed Field Electrophoresis System. After electrophoresis, gels were photographed on a UV-transilluminator. Lambed ladder PFG marker (LM) was used as size standards. (B) Increased formation of nuclear γ-H2AX foci in exposed K562. Cells were exposed to 10 μM 8-Cl-Ado and immunolabeled with anti-γ-H2AX antibodies and Rhodamine-conjugated anti-rabbit secondary antibodies (red). Nuclei were stained with Hoechst 33342 (blue). Cells were dropped on slides and viewed with Leica TCS SP2 confocal microscopy. Scale bar, 8 μm. (C) Western blotting analyses of γ-H2AX. After cell lysate proteins were separated by SDS-PAGE and transferred onto membrane, the blot was probed with anti-γ-H2AX antibodies. β-actin is used as control.

impact of 8-Cl-Ado and 8-Cl-ATP on DNA metabolism. In this study, we demonstrate that 8-Cl-ATP as an ATP analog inhibits Topo II-dependent relaxation and decatenation through inhibiting the hydrolysis of ATP and increasing the stability of closed clamp. Furthermore, 8-Cl-Ado can inhibit Topo II activities in exposed cells, thereby leading to decrease of DNA synthesis and to DNA double-stranded breaks (DSBs), which may contribute partly to 8-Cl-Ado-induced inhibitory proliferation.

We have previously demonstrated that 8-Cl-ATP, as an ATP analog, can interfere with ATP-dependent actin polymerization [30]. Because type II topoisomerases are ATP-dependent [7–11], we tested the effects of 8-Cl-Ado and 8-Cl-ATP on Topo II enzymes. We found that 8-Cl-ATP could inhibit Topo IIα-catalyzed relaxation (Fig. 2), decatenation (Fig. 5) and ATP hydrolysis (Fig. 3) *in vitro*. We also observed that 8-Cl-Ado exposure led to inhibition of Topo II activities *in vivo*, evidenced by the decatenation assays using a specific substrate for Topo II enzymes, the kinetoplast DNA (Fig. 1B). It is demonstrated that 8-Cl-Ado can convert into 8-Cl-ATP in living cells [25]. Therefore, we suggest that 8-Cl-Ado can inhibit Topo II enzymes through converting 8-Cl-ATP in exposed cells.

Since both ATP and 8-Cl-ATP can bind to Topo II enzymes, it is difficult to differentiate the forms of nucleotide-bound enzymes in the presence of both ATP and 8-Cl-ATP. Under this condition, three functional forms of Topo II enzymes might occur in the reactions. One is available enzyme using two ATP molecules, which functions normally. Another is inhibited enzyme bound two 8-Cl-ATPs, for example, the relaxation of supercoiled DNA could be abolished completely in the presence of 8-Cl-ATP alone (Fig. 2B, lane 9 and Fig. 2C, lane 4). The third is also inhibited enzyme that binds one ATP and one 8-Cl-ATP. Because 8-Cl-ATP can compete with ATP for the enzyme, inhibiting Topo II-catalyzed relaxation (Fig. 2B and C), which form of the enzymes to be predominant in the reactions depends on the molar ratios between ATP and 8-Cl-ATP. The ATP nonhydrolyzable analog, AMPPNP, has been shown to be able to stabilize closed clamp of human Topo II [35,43,44]. 8-Cl-ATP, just like AMPPNP, increased the formation of salt-stable closed clamp (Fig. 4) and inhibited the hydrolysis of ATP (Fig. 3). Therefore, it can be speculated that 8-Cl-ATP might act

in a similar manner with AMPPNP. A recent study demonstrated that binding of AMPPNP induces an enzyme conformation characterized by hindered DNA binding and release [13], which is consistent with the inhibition of ATP hydrolysis and the stabilization of closed clamp. Whether 8-Cl-ATP binding induces a similarly conformation and the mechanism of its act need to be investigated.

The mechanisms of the enzyme inhibition by 8-Cl-Ado in exposed cells must be more complicated than that of 8-Cl-ATP-inhibited Topo II activities *in vitro*. Three reasons why 8-Cl-Ado exposure can lead to decrease of the enzyme activities in cells might be considered. First, the inhibitory effect is resulted directly from increased 8-Cl-ATP and decreased ATP in 8-Cl-Ado-exposed cells, due to depletion of cellular ATP by 8-Cl-Ado-converted 8-Cl-ATP [25,26]. Alternatively, the decrease of Topo II activities in cells is attributed to the inhibition of enzymes by 8-Cl-ATP. Second, 8-Cl-ATP as an ATP nonhydrolyzable analog binds and increases the closed clamp conformation of the enzyme, leading to trap of the enzyme on chromosomal DNA. Therefore, the amounts of free enzyme that can be extracted into nuclear extracts will be decreased. Anyhow, the direct inhibition of enzyme activity and the decrease of free enzyme recovery both can be explained by enzyme inhibition by 8-Cl-Ado-converted 8-Cl-ATP. Third, 8-Cl-Ado exposure may decrease total amount of Topo II enzymes in targeted cells, since 8-Cl-ATP can inhibit RNA and protein syntheses [25–28]. In this case, the inhibition of enzyme activities in the nuclear extract we tested is actually ''decreased'' but not ''inhibited''. Among the three considerations any one cannot be excluded.

Topo II enzymes are necessary for chromosome segregation during mitosis in various species including mammals [45–47]. If Topo II function is blocked after chromosome condensation, the cells are arrested at metaphase and the chromatids fail to separate [48,49]. It is generally accepted that Topo II poisons can induce DNA and chromosome damage with subsequent G2 delay. However, ''true'' Topo II-catalytic inhibitors do not damage DNA and chromosome directly, because they cannot accumulate cleavable complex. An exception is ICRF-193, which has been characterized by stabilization of the circular clamp conformation [18] and subsequently shown to be able to cause both Topo II–DNA cross-links and specific Topo II-mediated DNA cleavages [50]. Exposure of human leukemic CEM cells to catalytic inhibitors such as merbarone and SN22995 result in G2 arrest, and then cells escape the G2 block to proceed into mitosis, where dividing failure leads to re-replication and polyploidy cells [51]. Interestingly, it has been reported that 8-Cl-cAMP can induce structural chromosome aberrations such as gaps, acentric breaks, centric rings, and Robertsonian translocations [31]. Consistently, we have previously observed that 8-Cl-Ado exposure induces G2/M arrest and chromosome segregation failure, resulting in binuclei and multinuclei accumulation [29], which is correlated to the disruption of the dynamic instability of microfilament and microtubule systems [30]. In the current study, we found that 8-Cl-Ado inhibited Topo II activities in exposed cells, which provides at least in part the underlying molecular mechanism for the observed aberrant chromosome.

We observed increased generation of DNA DSBs and γ-H2AX foci in 8-Cl-Ado-exposed K562 (Fig. 7). The generation of DSBs is not simply due to interfering with DNA by 8-Cl-dATP. This notion is supported by the observation that 8-Cl-Ado can convert into only 8-Cl-ATP but not 8-Cl-dATP in living cells [25]. The unique decatenating and unknotting activity of Topo II is essential to efficiently carry out segregation of daughter chromosomes after DNA replication [1]. Therefore, lacking Topo II-mediated decatenation, cells try to segregate intertwined sister chromatids during mitosis, chromosome breakage may occur. We showed the inhibition of decatenating activity of Topo II by 8-Cl-ATP *in vitro* (Fig. 5) and by 8-Cl-Ado in exposed cells (Fig. 1), which may explain the 8-Cl-Ado-induced DSBs in K562 cells (Fig. 7) and chromosome breakage [31]. DNA DSBs may also result from the replication failures, for example when replication encounters nicks [21,22]. The closed clamp form can represent an obstacle for the progression of the replication fork [20], and replication fork arrest is processed to DSBs and recombination [21,22]. Furthermore, 8-Cl-ATP inhibits RNA synthesis [25–27]. Because RNA primers are required for the synthesis of lagging strand, the lagging strand synthesis might be slowed down during 8-Cl-Ado exposure, resulting in uncoupling of leading and lagging strand replication, which is similar to stalled replication forks. Together, increase of closed clamp and inhibition of RNA synthesis appear to be account for replication fork arrest. We did find the reduction of BrdU incorporation into DNA (Fig. 6). At present we have no evidence for the linking between DSB generation and replication fork arrest, which is in progress.

In summary, 8-Cl-Ado exposure can inhibit Topo II activities by converting into 8-Cl-ATP in cells, thereby leading to DNA DSBs. This finding can explain the cell-cycle arrest, cell death and chromosome segregation aberration of cancer cells exposed to 8-Cl-Ado and 8-Cl-cAMP. In addition, our data suggest that 8-Cl-ATP as an ATP analog may be a useful tool in the studies of the enzymes and anti-Topo II enzyme drugs.

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