4-Nitroquinoline-1-Oxide Induces the Formation of Cellular Topoisomerase I-DNA Cleavage Complexes

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Abstract

RecQ helicase BLM-deficient cells are characteristically hypersensitive to 4-nitroquinoline-1-oxide (4NQO). We recently reported that isogenic BLM-deficient cells (PNSG13) are more sensitive than BLM-complemented cells (PNSF5) to camptothecin, which specifically traps topoisomerase I cleavage complexes (Top1cc). We now report that PNSG13 are also 3.5-fold more sensitive to 4NQO compared with PNSF5 and that 4NQO induces higher levels of Top1cc and reduced histone γ -H2AX in PNSG13 than in PNSF5. Similarly, 4NQO induces more Top1cc in primary fibroblasts from a patient with Bloom syndrome than in normal human fibroblasts. 4NQO also induces Top1cc in colon cancer HCT116 and HT29 cells in a time- and concentration-dependent fashion. Of note, distinct from camptothecin, the Top1cc produced by 4NQO accumulate progressively after 4NQO addition and persist following 4NQO removal. The Top1cc induced by 4NQO are detectable by alkaline elution. To examine the functional relevance of the Top1cc induced by 4NQO, we used two stable topoisomerase I small interfering RNA (siRNA) cell lines derived from HCT116 and MCF7 cells. Both topoisomerase I siRNA cell lines are resistant to 4NQO, indicating that Top1cc contribute to the cellular activity of 4NQO. Collectively, these data show that 4NQO is an effective inducer of cellular Top1cc. Because 4NQO does not directly trap Top1cc in biochemical assays, we propose that active metabolites of 4NQO trap Top1cc by forming DNA adducts. Induction of Top1cc and histone γ -H2AX by 4NQO may contribute to the cellular effects of 4NQO, including its selective activity toward RecQ helicase BLM-deficient cells. (Cancer Res 2006; 66(13): 6540-5)

Introduction

Nuclear DNA topoisomerase I is essential for relaxing DNA supercoiling generated during replication, transcription, repair, and recombination (1–4). Topoisomerase I-mediated DNA relaxation proceeds by inducing of single-strand breaks (SSB) that allows the broken DNA strand to rotate around the complementary intact strand and then by rapidly sealing the broken phosphate backbone to restore the DNA continuity. DNA cleavage is due to the formation of transient covalent topoisomerase I-DNA intermediates, which are called topoisomerase I cleavage complexes (Top1cc). Camptothecin and other anticancer topoisomerase I inhibitors, such as indolocarbazoles and indenoisoquinolines, trap Top1cc reversibly by

binding specifically at the interface of the topoisomerase I-DNA complexes (5, 6). Stabilized cleavage complexes are converted into irreversible DNA breaks (topoisomerase I covalent complexes), as replication or transcription complexes collide into the Top1cc. Exogenous and endogenous DNA lesions, such as 8-oxoguanosine, SSB, UV lesions, and carcinogenic adducts, can also trap Top1cc and thereby induce topoisomerase I-mediated DNA damage (7–11).

RecQ helicase-deficient cells (WRN and BLM in humans and Sgs1 in yeast) are hypersensitive to camptothecin (refs. 5, 12, 13; <http://discover.nci.nih.gov/pommier/pommier.htm>), and we recently found that BLM-deficient cells (PNSG13) are not only more sensitive to camptothecin but also form increased Top1cc in response to camptothecin compared with their BLM-complemented counterparts (PNSF5; ref. 14). BLM cells are also characteristically hypersensitive to 4-nitroquinoline-1-oxide (4NQO; refs. 15–20) with possible defects in apoptosis (21), but the mechanisms of this hypersensitivity have thus far remained unexplained. Genetic deficiency for the BLM RecQ helicase results in Bloom syndrome, a rare autosomal recessive genetic disease characterized by high frequency of sister chromatid exchanges (22) and predisposition to a wide variety of cancers occurring at an early age (23). The BLM protein functions in association with topoisomerase III α as a helicase-topoisomerase complex that can decatenate and resolve stalled replication forks (23).

The carcinogenic and mutagenic properties of 4NQO were first reported in 1957 (24). Its metabolic activation pathways have since been elucidated. 4NQO is metabolized into 4-acetoxyaminoquinoline-1-oxide (Ac-4HAQO), which can form covalent adducts to C⁸ or N² of deoxyguanine and N⁶ of deoxyadenine in DNA (25, 26). 4NQO also produces oxidative damage and DNA SSB (25, 27, 28). In the current study, we show that 4NQO effectively induces the formation of irreversible Top1cc with a greater frequency in BLM-deficient cells. These Top1cc are readily detectable in 4NQO-treated cells as protein-associated DNA SSB (PASB), which are characteristic of Top1cc (29), but cannot be formed with recombinant topoisomerase I in biochemical assays. We propose that 4NQO-induced DNA adducts trap Top1cc irreversibly and that these Top1cc are involved in the cellular effects of 4NQO, including the selective activity toward RecQ helicase BLM-deficient cells.

Materials and Methods

Drugs and enzymes. 4NQO, camptothecin, and etoposide (VP-16) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Each of these drugs was prepared as 10 mmol/L stock solutions in 100% DMSO. Aliquots were stored at -20°C , thawed, and diluted with complete medium just before each experiment. The final concentration of DMSO in culture medium did not exceed 0.1% (v/v). Recombinant human topoisomerase I was purified from TN5 insect cells (HighFive, Invitrogen Corp., San Diego, CA) using a baculovirus construct for the NH₂-terminal truncated human topoisomerase I cDNA as described previously (30). Recombinant human topoisomerase II α was expressed in yeast and purified as described (31).

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Cell culture. Human colon carcinoma HCT116 and HT29 cell lines were provided by the National Cancer Institute Developmental Therapeutics program (Dr. Nick Scudiero). Topoisomerase I small interfering RNA (siRNA) cells HCT116-siRNA-Top1 and MCF7-siRNA-Top1 and their respective control cell lines, which were established with HCT116 and human breast cancer MCF7 cell lines by stable topoisomerase I siRNA transfection, have been described elsewhere (32). Normal human skin fibroblast GM00037 cells and skin fibroblasts GM01492 from a patient with Bloom syndrome were obtained from Coriell Cell Repositories (Camden, NJ). Cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Grand Island, NY) at 37°C in a 5% CO₂ humidified atmosphere. Isogenic BLM-deficient PSNG13 and BLM-complemented PSNF5 fibroblasts (33) were generously provided by Dr. Ian Hickson (Oxford, United Kingdom) and cultured under the same conditions as the above, except for the addition of G-418 (340 µg/mL; Sigma-Aldrich) in the culture medium.

Assessment of cytotoxicity. Cytotoxicity of 4NQO was assessed by the sulforhodamine B (Sigma-Aldrich) assay (34). IC₅₀ was calculated using the software Prism 4 (GraphPad Software, Inc., San Diego, CA). Relative resistance, called resistance factor, was expressed as the ratio of the IC₅₀ of resistant cells to IC₅₀ of corresponding sensitive cells. Each experiment was done at least twice in triplicate.

Detection of topoisomerase I-DNA complexes. Covalent topoisomerase I-DNA cleavage complexes were detected by the immunocomplex of enzyme bioassay (32, 35). Briefly, 1×10^6 cells were lysed in 1% Sarkosyl and homogenized with a Dounce homogenizer. The cell lysates were gently layered on CsCl step gradients and centrifuged at $165,000 \times g$ at 20°C for 20 hours. Thirteen consecutive 0.5 mL fractions were collected, diluted with an equal volume of 25 mmol/L sodium phosphate (pH 6.6), and applied to Immobilon-P membranes (Millipore Corp., Billerica, MA) using a slot-blot vacuum manifold. Topoisomerase I-DNA complexes were detected by immunoblotting using the C21 topoisomerase I monoclonal antibody (a kind gift from Dr. Yung-Chi Cheng, Yale University, New Haven, CT) or the topoisomerase II polyclonal antibody from the Abcam, Inc. (Cambridge, MA). All experiments were done independently at least twice.

Confocal microscopy of γ -H2AX foci formation. Cells used for microscopic analysis were grown in Nunc chamber slides (Nalgen, Rochester, NY) using 0.5 mL growth medium. For γ -H2AX foci detection, cells were fixed and permeabilized as described previously using 4% paraformaldehyde and cold 70% ethanol (14). Nonspecific binding was blocked using 8% bovine serum albumin in PBS. Fixed cells were stained overnight with anti-mouse monoclonal primary antibodies (in 1% bovine serum albumin at 4°C; Upstate Technology, Charlottesville, VA) and tagged with fluorescent secondary antibodies (Molecular Probes, Inc., Carlsbad, CA) for 2 hours at room temperature. Slides were mounted using Vectashield mounting liquid (Vector Laboratories, Burlingame, CA) and sealed. Prepared slides were visualized using a Nikon (Melville, NY) Eclipse TE-300 confocal laser scanning microscope system and stored as TIFF images.

Alkaline elution assays. Alkaline elution assays were used to quantitate DNA SSB and to estimate DNA-protein cross-links, from which the production of PASB was inferred (36–38). Cellular DNA was radiolabeled with 1 µCi/mL [³H]thymidine (Perkin-Elmer Life Science Co., Boston, MA) for 48 hours at 37°C and chased in nonradioactive medium overnight. After drug treatments, cells were scraped in HBSS and counted to determine the appropriate loading. Aliquots were placed in drug-containing ice-cold HBSS. After alkaline elution, filters were incubated at 65°C with 1 mol/L HCl for 45 minutes and 0.04 mol/L NaCl was added for an additional 45 minutes. Radioactivity in all fractions was measured with a liquid scintillation analyzer (Packard Instruments, Meriden, CT). DNA-protein cross-links were analyzed under nondeproteinizing, DNA-denaturing conditions using protein-adsorbing filters (29, 36, 38). SSB were assessed under deproteinizing, DNA-denaturing conditions using filters that do not adsorb proteins (polycarbonate filters; refs. 36, 38). SSB frequencies were calculated as $[\log(R_t / R_0) / \log(R_3 / R_0)] \times 300$, where R_t , R_0 , and R_3 correspond to the DNA selections for 4NQO-treated cells, untreated cells, and cells treated with 3 Gy, respectively.

Induction of topoisomerase I and II cleavage complexes in cell-free systems. To test whether 4NQO directly trapped topoisomerase I or II, recombinant topoisomerase I and II enzyme cleavage assays were done as described (9, 39). Imaging was done using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western blot analyses. Topoisomerase I levels in topoisomerase I siRNA cell lines and the corresponding control cell lines and in the BLM-deficient and BLM-proficient cells were measured by Western blotting with the C21 topoisomerase I monoclonal antibody. Protein bands were quantified with the software from Li-Cor Bioscience (Lincoln, NE). The experiments were done at least twice independently, and data are expressed as mean $\pm$ SD.

Results

Differential cytotoxicity, Top1cc, and γ -H2AX foci induced by 4NQO in BLM-deficient and BLM-proficient cells. We recently reported that BLM-deficient cells (PSNG13) are more sensitive to camptothecin than isogenic BLM-complemented cells (PSNF5; ref. 14). Figure 1A shows that PSNG13 are also 3.5-fold more sensitive to 4NQO than PSNF5. The IC₅₀ for PSNG13 cells was 49.4 ± 4.7 nmol/L, whereas the BLM-complemented cells exhibited an IC₅₀ of 173.8 ± 15 nmol/L. To detect whether 4NQO can induce Top1cc, we used the immunocomplex of enzyme bioassay (14, 35). 4NQO produced Top1cc in both PSNG13 and PSNF5 (Fig. 1B). However, PSNG13 produced more Top1cc in response to 4NQO than PSNF5 (Fig. 1B). Top1cc were 2.62-fold higher in PSNG13 than in PSNF5 at 10 µmol/L 4NQO and 1.57-fold higher at 30 µmol/L 4NQO (Fig. 1B). 4NQO also induced ~2-fold higher frequency of Top1cc in the untransformed, primary GM01492 fibroblasts from a patient with Bloom syndrome than in primary normal human GM00037 fibroblasts (Fig. 1C). To test whether the difference in topoisomerase I trapping could be attributed to different topoisomerase I expression levels, we assayed topoisomerase I protein levels using Western blotting of whole-cell extracts. The level of topoisomerase I protein in the BLM-deficient and BLM-proficient cells were comparable (Fig. 1A) and thus could not account for the greater Top1cc in the BLM-deficient cells. These results show that 4NQO induces Top1cc in all four cell lines examined and that BLM-deficient cells tend to form more Top1cc that are not due to differences in global topoisomerase I protein. We further examined the formation of γ -H2AX foci, which are formed in response to Top1cc (14, 40), and found that treatment of PSNG13 (BLM-deficient) cells with 4NQO also produced reduced γ -H2AX compared with PSNF5 (BLM-proficient) cells (Fig. 1D) as shown previously with camptothecin in the same cell lines (14).

4NQO induces irreversible Top1cc in a time- and concentration-dependent manner. Using camptothecin as the positive control, we examined the features of Top1cc induction by 4NQO. In human colon carcinoma HCT116 cells, 4NQO also induced the formation of Top1cc in a time- and concentration-dependent fashion (Fig. 2A). Relatively few Top1cc were detectable after 30-minute exposure to 4NQO and Top1cc were readily detectable after 1 or 3 hours with levels comparable with 5 µmol/L camptothecin (Fig. 2A). One-hour exposures at various 4NQO concentrations showed that Top1cc were easily detectable at concentrations above 10 µmol/L 4NQO in two human colon carcinoma cells (HCT116 and HT29; Fig. 2A and B).

Camptothecin-induced Top1cc are characterized by their reversibility on drug removal (29, 41). Accordingly, a 1-hour postincubation of both HT29 (Fig. 2B) and HCT116 (data not shown) cells after camptothecin removal drastically reduced the Top1cc. In striking contrast, the 4NQO-induced Top1cc remained

Figure 1. Differential sensitivity, induction of topoisomerase I-DNA complexes, and γ -H2AX foci by 4NQO in BLM-deficient and BLM-proficient cells. **A**, survival curves of BLM-deficient PSNG13 and BLM-complemented PSNF5 cells exposed to 4NQO for 72 hours were determined by the sulforhodamine B assay. Experiments were conducted twice in triplicate independently. Points, mean; bars, SD. Basal levels of topoisomerase I protein in BLM-deficient (PSNG13 and GM01492) and BLM-proficient cells (BLM-complemented PSNF5 and normal GM00037 fibroblasts) were detected by Western blotting. **B**, induction of topoisomerase I-DNA complexes by 4NQO in BLM-deficient (PSNG13) and BLM-complemented (PSNF5) cells detected by the immunocomplex of enzyme bioassay. Quantitation of the results was conducted with PhotoShop 7.0. Values were calculated as (total pixel intensity of the five bands corresponding to fractions 6-10) – (total pixel intensity of the corresponding bands in the absence of 4NQO). **C**, as in (**B**) for the primary Bloom syndrome fibroblasts (GM01492) and normal fibroblasts (GM00037). **D**, reduced formation of γ -H2AX foci in BLM-deficient cells treated with 4NQO. Exponentially growing PSNG13 (BLM-deficient) or PSNF5 (BLM-complemented) cells were exposed to 10 or 10 mmol/L 4NQO for 1 hour. After slide preparation as described in Materials and Methods, cells were studied for formation of microscopic nuclear foci corresponding to phosphorylated histone H2AX, γ -H2AX. Color images were converted to grayscale, and nuclear outlines were superimposed using corresponding propidium iodide images. Representative images from independent experiments.

stable even for 3-hour incubation following 4NQO removal (Fig. 2B), indicating the irreversibility of the Top1cc induced by 4NQO.

Additionally, 4NQO did not produce any detectable covalent Top2cc in HCT116 or HT29 under conditions where Top1cc

were readily detected (Fig. 2C). These experiments show that 4NQO induces irreversible Top1cc but is ineffective against topoisomerase II.

4NQO generates PASB in cells. Protein-linked DNA breaks are characteristic of topoisomerase cleavage complexes (3, 36). DNA SSB mediated by topoisomerases can be detected by alkaline elution only under deproteinizing conditions (29, 42) as each break is associated with a topoisomerase molecule (38). This is in contrast with frank breaks, such as those induced by ionizing radiation that can be similarly detected without or with deproteinization (36). We used the alkaline elution to detect whether 4NQO induced SSB and whether these breaks corresponded to PASB (29). Alkaline elution is a sensitive and quantitative method for the detection of SSB (38). 4NQO induced high SSB frequency (~ 900 rad equivalent SSB) at 3 μ mol/L in HCT116 (Fig. 3). Deproteinizing conditions were required to detect these breaks, which was not the case for 3 Gy (Fig. 3A). These results are consistent with the induction by 4NQO of PASB in the form of Top1cc.

Exposure of HCT116 cells to increasing concentrations of 4NQO for 1 hour and quantitation of independent alkaline elution experiments showed that 4NQO induces increasing SSB at

Figure 2. 4NQO produces irreversible Top1cc but no Top2cc. **A**, time course and concentration dependence of Top1cc induction by 4NQO in HCT116 cells. Camptothecin was used as a positive control. **B**, persistence of the Top1cc induced by 4NQO in HT29 cells on 4NQO removal for 1 or 3 hours. Note the reversibility of camptothecin-induced Top1cc. **C**, 4NQO does not produce Top2cc. VP-16 was used as a positive control for Top2cc formation.

Figure 3. 4NQO induces PASB. SSB and non-PASB produced in HCT116 cells treated with 4NQO for 1 hour were detected by the alkaline elution assay. **A**, representative alkaline elution experiment showing total SSB (measured under deproteinizing conditions; *closed symbols*) and non-PASB (measured under nondeproteinizing conditions; *open symbols*) in cells exposed to 3 μmol/L 4NQO for 1 hour. **B**, dose response for SSB (*closed symbols*) and non-PASB (*open symbols*) induced by 4NQO. Alkaline elution results are expressed in rad equivalents as described in Materials and Methods. Data at each concentration of 4NQO represent two independent determinations.

concentration below 3 μmol/L and that SSB reach a plateau at and above 3 μmol/L (Fig. 3B). As the 4NQO concentration increased, 4NQO induced detectable SSB under nondeproteinizing conditions, but this signal was much lower than the SSB signal under deproteinizing conditions (Fig. 3B, *open symbols*). This result is consistent with the production of PASB by 4NQO together with a smaller component of protein-free SSB at high concentrations.

4NQO does not directly inhibit topoisomerase I or II enzymes. To test whether induction of cellular Top1cc by 4NQO is due to a direct effect on topoisomerase I enzyme, we did biochemical analyses with recombinant human topoisomerase I enzyme. In this system, camptothecin was used as a positive control to show the direct trapping of Top1cc (Fig. 4A, lane 3). However, 4NQO, even at a high concentration of 100 μmol/L, produced a comparable profile of bands as topoisomerase I enzyme alone, which shows lack of direct topoisomerase I inhibition by 4NQO (Fig. 4A). Similarly, no cleavage was observed for Top2cc under conditions where VP-16 produced Top2cc. These experiments show that 4NQO does not act as a direct topoisomerase inhibitor (Fig. 4B).

Topoisomerase I-deficient cells are resistant to 4NQO. To examine the functional relevance of the Top1cc induced by 4NQO, we used two stable topoisomerase I siRNA cell lines derived from HCT116 and from breast cancer MCF7 cells. Both HCT116-siTop1 and MCF7-siTop1 cell lines express ~20% topoisomerase I protein compared with their respective control cell lines (Fig. 5A, *insets*). Both HCT116-siTop1 and MCF7-siTop1 cell lines displayed ~2-fold resistance to 4NQO compared with their corresponding control siRNA cell lines (Fig. 5A). We further compared the ability of 4NQO to form γ-H2AX foci in MCF-7-siTop1 and MCF-7-siRNA control cells. Figure 5B and C revealed that although both cell lines produced γ-H2AX foci kinetically in response to 4NQO treatment the net increase of γ-H2AX foci in the topoisomerase I siRNA cells was significantly less than that in the corresponding control siRNA cells. These results show that the decreasing topoisomerase I protein level rescues the cells from the cell killing and DNA damage by 4NQO, which indicates that Top1cc contributes to the cellular toxicity of 4NQO.

Discussion

Top1cc result from the covalent binding of the catalytic tyrosyl group (human Tyr⁷²³) of the enzyme to the phosphate group at the 3'-terminus of the cleaved DNA strand at the moment and site that topoisomerase I enzyme attacks and breaks one strand of duplex DNA (3, 5, 43). The covalent complexes reverse as the enzyme religates the broken DNA. Thus, Top1cc are the key intermediates by which topoisomerase I exerts its key DNA cleavage and religation activities. Under physiologic conditions, the existence of such complexes is fundamental to the basic function of topoisomerase I. As they are very transient, Top1cc are practically undetectable under physiologic conditions. Many exogenous and endogenous factors can reversibly or irreversibly trap topoisomerase I complexes and convert them into lethal intermediates as they lead to persistent DNA breaks and covalent topoisomerase I-DNA adducts (refs. 5, 12; <http://discover.nci.nih.gov/pommier/pommier.htm>).

In the current study, we report for the first time that the potent carcinogen and mutagen 4NQO effectively produces irreversible Top1cc. 4NQO elicits time- and concentration-dependent Top1cc that persist for >3 hours following 4NQO removal (Figs. 1 and 2). Alkaline elution assays further indicated the presence of cellular Top1cc as PASB, which are characteristic of topoisomerase

Figure 4. 4NQO does not produce topoisomerase I (A) or topoisomerase II (B) cleavage complexes in a cell-free system. Reactions were done at the indicated 4NQO for 20 minutes at 30°C. Camptothecin (CPT) and VP-16 were used as positive control for topoisomerase I and II, respectively.

Figure 5. Reduction of topoisomerase I expression leads to partial resistance to 4NQO and reduced formation of γ -H2AX foci by 4NQO. **A**, survival curves of HCT116-siTop1 MCF7-siTop1 and their respective control siRNA cells. The cytotoxicity of 4NQO in two pairs of topoisomerase I siRNA tumor cell lines was determined by the sulforhodamine B assay. **Insets**, topoisomerase I expression in the topoisomerase I siRNA cell lines and their control cell was determined by Western blotting with β -actin as loading control. **B**, reduced formation of γ -H2AX foci in MCF-7-siTop1 cells treated with 4NQO. Cells were exposed to 50 nmol/L 4NQO for 15, 30, and 60 minutes. After slide preparation as described in Materials and Methods, cells were studied for formation of microscopic nuclear foci corresponding to phosphorylated histone, γ -H2AX. Color images were converted to grayscale, and nuclear outlines were superimposed using corresponding propidium iodide images. Representative experiments. **C**, percentage of γ -H2AX foci-positive cells and average foci per γ -H2AX-positive cell. At least 100 cells for each experimental group were counted.

cleavage complexes (29, 36). Increasing exposure to 4NQO also produced some frank breaks (Fig. 3). Collectively, our data show that 4NQO is an effective inducer of Top1cc in all human cell lines examined: in the four transformed cell lines [PSNG13, PSN15 (Fig. 1), HCT116, and HT29 (Figs. 2 and 3)] and in the two primary fibroblasts [GM00037 and GM01492 (Fig. 1)]. In contrast, 4NQO does not seem to affect topoisomerase II (Fig. 2C).

The induction of irreversible Top1cc by 4NQO (Fig. 2) is not the consequence of a direct action of 4NQO on topoisomerase I as topoisomerase I cleavage activity is unaffected by 4NQO in cell-free system (Fig. 4). 4NQO is carcinogenic only after being metabolically activated (including via 4NQO reductase, EC1.7.1.9 in cytosol) to its ultimate carcinogen Ac-4HAQO (Fig. 6; refs. 25, 26, 44). Two

active metabolites of Ac-4HAQO form three main carcinogenic DNA monoadducts: two on guanine (dG-C⁸-AQO and dG-N²-AQO) and one on adenine (dA-N⁶-AQO; Fig. 6; ref. 25). It is well established that Top1cc can be trapped or induced by a wide variety of carcinogenic adducts at guanine N² [benzo[*a*]pyrene (30), benzo[*c*]phenanthrene (30), and acetaldehyde adducts (9)], guanine O⁶ [methyl adducts (45)], and adenine N⁶ [ethyl adducts (46); reviewed in ref. 7 and updated at <http://discover.nci.nih.gov/pommier/pommier.htm>]. It is therefore plausible that the DNA monoadducts produced by 4NQO also elicit Top1cc. Because there were no necessary enzymes and other suitable physiologic conditions for 4NQO transformation in our cell-free system, 4NQO itself did not trap Top1cc as camptothecin did (Fig. 4).

Previous studies have indicated that SSB are induced *in vitro* and *in vivo* by 4NQO (47–50). Our experiments suggest that these breaks are mainly PASB, which probably correspond to Top1cc (Fig. 3). We found that 4NQO also induces “frank breaks” [i.e., non-protein-linked SSB that are detectable under nondeproteinizing conditions (Fig. 3; ref. 36)]. Such frank breaks are less frequent than the PASB and increase with 4NQO concentration (Fig. 3). Whether such frank breaks contribute to the formation of Top1cc (51) or are a consequence of the irreversible Top1cc or other DNA lesions remains to be determined.

The finding of induction of Top1cc by 4NQO sheds some light on the cellular and biological effects of 4NQO. These Top1cc must contribute to the cellular damage and lethality resulting from 4NQO exposure as shown by the 2-fold resistance of cell lines with topoisomerase I down-regulation (Fig. 5) and by the greater sensitivity and increased Top1cc in BLM-deficient cells (Fig. 1). Thus, the formation of greater Top1cc in BLM-deficient cells provides a rationale for the hypersensitivity of Bloom syndrome

Figure 6. Schematic representation for the proposed mechanism and consequence of the 4NQO-induced formation of covalent Top1cc.

cells to 4NQO (Fig. 1). The finding that 4NQO generates TopIcc also offers a novel perspective on its molecular mechanisms of cytotoxicity, carcinogenicity, and mutagenicity. As 4NQO-induced DNA damage is an early event in its carcinogenesis (52, 53) and irreversible TopIcc induce recombinations (54), the covalent TopIcc produced by 4NQO may play a role in 4NQO carcinogenicity.

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