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Inhibition of Topoisomerase II Activity in Repair-Proficient CHO K1 Cells by 2-[(Aminopropyl)Amino]Ethanethiol (WR-1065)[1]

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[3]The abbreviations used are: WR-2721, *S*-2-(3-aminopropylamino)ethylphosphorothioic acid; WR-1065, 2-[(aminopropyl)amino]ethanethiol; topo I, type I topoisomerase; topo II, type II topoisomerase; DMF, dose modifying factor; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; kDa, kilodalton.

ABSTRACT

The aminothiol 2-[(aminopropyl)amino]ethanethiol (WR-1065) is the active thiol of the clinically studied radioprotective agent *S*-2-(3-aminopropylamino) ethylphosphorothioic acid (WR-2721). WR-1065 is an effective radiation protector under in vitro conditions when it is administered 30 min prior to radiation exposure at a concentration of 4 mM to repair-proficient Chinese hamster ovary K1 cells (i.e., a dose modification factor of 1.4). In contrast, the DNA double-strand break, repair-deficient Chinese hamster ovary xrs-5 cell line is not protected under these conditions (i.e., a dose modification factor of 1.0). Topoisomerase (topo) I and II activities and protein contents were measured in both K1 and xrs-5 cell lines and were found to be similar in magnitude. Neither exposure to radiation, to WR-1065, or to both affected these variables in xrs-5 cells. WR-1065 was effective, however, in reducing topo II activity by a factor of 2 in the repair-proficient K1 cell line. Topo II protein content, however, was not affected by these exposure conditions. One of several mechanisms of radiation protection attributed to aminothiol compounds has been their ability to affect enzymatic reactions involved in DNA synthesis, repair, and cell cycle progression. These results demonstrate a modifying effect by 2-[(aminopropyl)amino]ethanethiol on a specific nuclear enzyme (i.e., type II topoisomerase), which is involved in DNA synthesis. These results also suggest that differences do exist between the topo II enzymes isolated from the parent repair-proficient K1 and the DNA double-strand break, repair-deficient xrs-5 mutant cell lines.

INTRODUCTION

The phosphorothioate WR-2721[3] has been investigated in clinical trials as an adjuvant for use with radiation (1,2) and chemotherapy (3–5) to reduce normal tissue toxicity. WR-2721 and its free thiol WR-1065 have also been reported to be effective in protecting against radiation- or chemotherapy-induced mutagenesis (6–10) and carcinogenesis (11–13). With respect to the antimutagenic effect, these agents significantly reduced the frequency of mutations at the *hprt* locus in cultured mammalian cells and rodent T splenocytes, even when they were administered up to 3 h following exposure of cells to radiation (6,10).

While the major mechanisms proposed to describe the protective effects of this class of aminothiols have focused on their well-characterized physiochemical properties (14), they have been implicated in affecting DNA synthesis and repair (15,16), DNA nuclease activity (17), and cell cycle progression (18,19) in mammalian cells. As early as 1967, it was proposed that protection by aminothiol compounds is mediated via inherent cellular DNA repair processes (20). More recently it has been reported that the disulfide form of WR-1065 can enhance the topoisomerase-I-mediated unwinding of supercoiled plasmid pIBI30 DNA (21). It has been suggested that this result was not unexpected due to the similarity in chemical structure between the disulfide form of WR-1065 and the polyamine spermidine, a polyamine which has been reported to be effective in enhancing the relaxation of DNA supercoils via topoisomerase reactions (22).

Using repair-proficient and -deficient mammalian cell lines, we have extended studies on the role of aminothiol-mediated effects on DNA-associated enzymes. In particular, we have characterized the effects of WR-1065 (the free thiol form of WR-2721) exposure on topo I and II activities in CHO repair-proficient K1 and repair-deficient xrs-5 cell lines (23).

MATERIALS AND METHODS

Cells and Culture Conditions. CHO K1 and xrs-5 cell lines were maintained as stock cultures in α–Minimal Essential Medium (Gibco) with 10% fetal calf serum (Biologos) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

Drug Treatment. WR-1065 used in these studies was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The working solution of WR-1065 was made up in PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 2.6 mM KCl) buffer at a 1 M concentration and sterilized by filtration immediately before use. Cells were exposed to WR-1065 at a final concentration of 4 mM. At that concentration, WR-1065 is nontoxic and is maximally effective as an antimutagen and a radioprotector (6-8).

Survival Studies. Exponentially growing cultures of K1 and xrs-5 cells were irradiated with 50-kVp x-rays either in the presence or absence of WR-1065 (4 mM). All experiments were performed in triplicate. Cell survival was determined by plating appropriate numbers of cells to give between 80 and 200 colonies per dish, 6 dishes per experimental point. The D_0 and 95% confidence limits were determined for each survival curve using a computer-fitted, least-squares regression model. Dose modification factors were determined from survival curves by comparing the corresponding D_0 values obtained for each of the WR-1065 conditions with that of the associated radiation-only controls.

Preparation of Nuclear Extracts. Exponentially growing CHO K1 or xrs-5 cells were used in all experiments. Following exposure to radiation and/or WR-1065, treated and control cells in plastic petri dishes were placed on ice, and a volume (equal to that of the culture medium) of cold solution containing 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, and 0.25 M sucrose was added. The cells (about 10^8) were centrifuged and then homogenized.

The nuclei were isolated as described by Taudou et al. (24) and extracted as described by Champoux and McConaughy (25), except that immediately after the washing of cells, a solution comprised of 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 50 μg/ml leupeptin, 1 μg/ml pepstatin, and 20 μg/ml aprotinin was added. The protein content of the nuclear and the cellular extracts was determined by the Bradford method (26). Glycerol was added to a final concentration of 30%, and the extracts were stored at −20 °C. These preparations served as the source for topo I and II activity, as well as for topo II immunoblotting. For the unknotting assay (which detects topo II catalytic activity), serial dilutions were made such that the reaction volumes of 20 μl contained a range of 50–800 μg of nuclear extract. For the DNA relaxation assay (which detects topo I activity in the absence of ATP), reaction volumes of 20 μl containing a range of 1–100 μg of nuclear extract were used.

Unknotting Assay for the Determination of Topo II Activity. The enzyme sources for this assay were from serial dilutions of nuclear extracts. The substrate used was knotted DNA that had been isolated from tailless capsids of the bacteriophage P4 Vir1 del10, according to a modification of the methods by Liu et al. (27). Reaction mixtures of 20 μl contained 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 40 μg/ml bovine serum albumin (nuclease free), and 1 mM ATP. The reactions were started by the addition of 0.6 μg of knotted DNA and terminated by the addition of 5 μl of a stop solution containing 5% SDS, 50 mM EDTA, 25% Ficol, and 0.05 mg/ml bromophenol blue. Samples were loaded on 0.8% agarose gels and electrophoresed at 1.5 V/cm for 15 h in Tris/Borate/EDTA buffer. Gels were stained in 1 μg/ml ethidium bromide and then destained and photographed over a UV light source. Quantitative determination of topo II activity was determined by densitometric measurement of photographic negatives. DNA that remained knotted migrated as

a single band to the top of the gels. One unit of unknotting activity is defined as the amount of enzyme that converts 50% of the substrate (knotted DNA) into the reaction product (unknotted DNA).

Relaxation Assay for the Determination of Topo I Activity. The substrate used in this assay was pUC8 plasmid DNA (90% supercoiled). Each reaction volume of 20 μl contained 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, 1 mm dithiothreitol, 0.1 mM EDTA, and 30 μg/ml bovine serum albumin (nuclease free). Serial dilutions of the nuclear extracts served as the source of topo I. The reactions were started by the addition of 0.6 μg of the supercoiled plasmid DNA. Following 30-min incubations at 37 °C, the reactions were terminated by the addition of 5 μl of a stop solution containing 50% sucrose, 2% SDS, 0.2 m EDTA, and 0.05% bromophenol blue. Samples were loaded in 0.8% gels, electrophoresed in Tris/Borate/EDTA buffer, stained, and photographed. Densitometric determination of the supercoiled form provided the means for quantitation. One unit of relaxation is defined as the amount of enzyme that converts 50% of the substrate (supercoiled DNA) into the reaction product (relaxed DNA). The omission of ATP from the assay eliminated the interference of topo II activity (28).

Immunoblotting for Determining the Topo II Protein Levels. Protein content of nuclear and cellular extracts were routinely determined (26). Extracts containing 100 μg of protein were analyzed in 8% SDS polyacrylamide gels (29). Proteins were transferred electrophoretically to nitrocellulose membranes and incubated with an anti-topo-II antibody recognizing the carboxyl terminal portion of the human topo II. This polypeptide was produced by an expression plasmid p56 z11–1.8 (30). The secondary antibody was anti-rabbit igG. Detection of the bands (corresponding to the 170 kDa form of topo II) was with the peroxidase reaction (Sigma) using 4-chloro-l-naphthol (Sigma) as the color indicator.

RESULTS

WR-1065 Effects on Radiation Response of K1 and xrs-5 Cells. Treatment of K1 cells with 4 mM of WR-1065 for 30 min prior to their exposure to 50-kVp x-rays resulted in significant radiation protection, as evidenced by an increase in the survival curve parameter D_0 (i.e., a measure of the terminal slope of the curve) from 2.35 ± 0.15 Gy to 3.35 ± 0.25 Gy (Figure 1). The D_0 values and associated 95% confidence limits were based on a computer-fitted least-squares regression model (6). A DMF of 1.4 was determined by taking the ratio of D_0 values from the survival curves describing the response of K1 cells in the presence and absence of WR-1065. In contrast, no protection against radiation-induced lethality by WR-1065 was evidenced for xrs-5 cells (Figure 1). The D_0 values from xrs-5 survival curves, under exposure conditions with or without WR-1065, were 0.70 ± 0.04 and 0.70 ± 0.09, respectively.

Figure 1 →

WR-1065 Effects on the Activities of Topoisomerases I and II. Topo I and II activities were measured in untreated K1 and xrs-5 cells along with (a) cells exposed to WR-1065 at a concentration of 4 mM for 30 min, (b) cells exposed to 10 Gy of ionizing radiation, and (c) cells exposed to WR-1065 and ionizing radiation. Presented in Figure 2 are data from a representative experiment describing topo II (panel A) and topo I (panel B) activities in nuclear extracts of K1 cells. Described in panel A are the activities of protein extracts from untreated or WR-1065-exposed K1 cells: 80 ng in lane 1, 40 ng in lane 2, 20 ng in lane 3, 10 ng in lane 4, and 5 ng in lane 5.

Figure 2 →

Topo I activity from nuclear extracts of 100 ng (lane 1), 30 ng (lane 2), 10 ng (lane 3), 3 ng (lane 4), 1 ng (lane 5), and no extract (−) are described in panel B for control and WR-1065-treated K1 cells. Densitometric data from four replicate experiments describing topo I and

topo II activities from K1 and xrs-5 cells are summarized in Table 1. No significant differences in topo I and II activities were observed between K1 and xrs-5 cells (all groups compared to K1 controls, $p \geq 0.05$, Student's two-tailed test). WR-1065 exhibited no effect on topo I activity in either of the two cell lines (all groups compared to K1 controls, $p \geq 0.5$), nor did it affect topo II activity in xrs-5 cells ($p \geq 0.5$, all groups compared to xrs-5 control cells). Topo II activity was, however, significantly reduced in the repair-proficient K1 cell line following exposure to WR-1065, as compared to the untreated control ($p = 0.019$). Following irradiation and exposure to WR-1065, topo II activity was somewhat reduced; the level of significance, however, is only suggestive (p value of 0.061).

Table 1 →

Since WR-1065 only affected topo II activity in the repair-proficient K1 cell line, topo II protein content was also determined for K1 cells exposed to WR-1065. Following exposure to 4 mM of WR-1065 for 30 min, exponentially growing K1 cells were lysed, and protein from nuclear extracts were electrophoresed in 8% SDS polyacrylamide gels and transferred to nitrocellulose. Blots were incubated with anti-topo-II antibody, and resulting data are presented in Figure 3. The molecular weights presented on the right ordinate are those of topo II (MW 170,000) and its proteolytic products. Prestained standards with associated molecular weights are also presented on the left side ordinate. Lanes 1–4 contain proteins from K1 cells, while lanes 5–8 contain proteins from xrs-5 cells. Untreated control cells are represented in lanes 1 and 5, WR-1065-treated cells are in lanes 2 and 6, irradiated cells are in lanes 3 and 7, and irradiated cells treated with WR-1065 are in lanes 4 and 8. A proteolytic band with a molecular weight of 150 kDa is evident only from K1 cells. Densitometric measurements of protein content (150 kDa and 170 kDa bands) are presented in Table 2 for comparison. No significant

differences in topo II protein content were observed between any of the experimental groups ($p \geq 0.30$).

Figure 3 & Table 2 →

Determinations of topo II protein contents in cellular lysates of K1 and xrs-5 cells were also performed, and data from a representative experiment are presented in Figure 4. Under these conditions, only a single major band of topo II was observed for both K1 and xrs-5 cells, suggesting that the minor band at 150 kDa observed for K1 nuclear isolates was due to enhanced proteolytic activity by this cell line. Under these conditions, the topo II protein content in K1 and xrs-5 cells was not significantly different ($p \geq 0.5$).

Figure 4 →

DISCUSSION

Aminothiols such as WR-1065, cysteamine, or glutathione have been reported to be effective in inhibiting DNA synthesis (15), strand rejoining (21), nuclease activity (17), and cell-cycle progression (19) in mammalian cells. These effects on cellular enzymatic processes have led investigators to propose that one possible mechanism of aminothiol protection involves the enhancement of endogenous DNA repair processes (31).

Radiation protection studies performed on DNA repair-deficient organisms strongly support the suggestion that endogenous repair systems are dominant parameters which can influence the magnitude of aminothiol-mediated protection. While cysteamine was found to be an effective protective agent in wild-type *E. coli*, it was not protective in bacterial strains with defects in the *rec* system (32,33). *E. coli* mutants deficient in poL I or UV endonucleases were also not amenable to protection by cysteamine (33).

X-ray repair-deficient *rad* mutants of *Saccharomyces cerevisia* were also reported to be unprotectable by cysteamine (34). Protection by cysteamine against radiation damage was

observed only in diploid wild-type yeast. Failure to observe protection in wild-type haploid or mutant *rad* diploid yeast suggests that radioprotection by this agent is mediated through a recombination-like mechanism (34). These findings have now been extended to mammalian cells by our demonstration that the repair-deficient xrs-5 CHO cell line is not amenable to protection against x-ray toxicity by the aminothiol WR-1065 (see Fig. 1, DMF = 1). In contrast, WR-1065 protected the wild type cell line, K1, by a factor of 1.4.

The aminothiols exhibit close structural similarities to polyamines, which are endogenous polybasic molecules having an affinity for DNA (35). The disulfide form (designated WR-33278) of the free thiol WR-1065 has the structure $H_2N-(CH_2)_3-NH-(CH_2)_2-S-S-(CH_2)_2-NH-(CH_2)_3-NH_2$, which is very similar to the structure of spermine (i.e., $H_2N-(CH_2)_3-NH-(CH_2)_2-(CH_2)_2-NH-(CH_2)_3-NH_2$). It has been reported that WR-2721 and its metabolites WR-1065 and WR-33278 can act as effective substrates and compete with polyamines for uptake into rat lung slices via well characterized polyamine transport processes (36). These compounds are also effective substrates for polyamine oxidase activity (37). Both classes of agents can interact directly with and bind to DNA (38,39).

Interest in assessing the effect(s) of the aminothiol WR-1065 on enzymatic activities in repair-proficient and -deficient cells was prompted in part by these considerations. The focus of this study was limited to an investigation of topo I and II because these enzymes are known to be important in DNA synthesis (40,41), and work with the polyamine spermine has been observed to enhance the formation of a stable nonconvalent complex between mammalian topo II and DNA (42). In contrast to an earlier report in which a cell-free system was used to demonstrate that the disulfide form (WR-33278) of WR-1065 was effective in stimulating the

eukaryotic topo I unwinding of negatively supercoiled DNA (21), we were unable to observe any effect of WR-1065 at a concentration of 4 mM on topo I activity in either K1 or xrs-5 cells.

In agreement with an earlier report (43), no differences in topo II activity were observed between control (untreated) K1 and xrs-5 cells. Likewise, no difference in topo II levels were measured either in nuclear or rapid cellular lysates of K1 and xrs-5 cells (see Figs. 3 and 4). When nuclear lysates were used to measure topo II content, a 150-kDa band was observed only for K1 cells. The absence of this band in xrs-5 derived nuclear lysates suggests a greater degree of proteolytic activity in K1 as compared to xrs-5 cells. The use of rapid cellular lysates demonstrated the presence of only one topo II band at 170 kDa for both cell lines (see Fig. 4).

Topo I and II activities were unaffected in both K1 and xrs-5 cell lines following exposure to ionizing radiation. However, WR-1065 was effective in inhibiting topo II activity, but only in K1 cells. The implications of this phenomenon are at present unclear. The ability of a relatively high concentration (i.e., 4 mM) of WR-1065, which is required for maximal radiation protection, to inhibit topo II activity in K1 but not xrs-5, suggests there are differences between the cell lines with respect to their topo II systems. If WR-1065 or its disulfide are behaving similar to a polyamine such as spermine, these results might suggest subtle differences in enzyme structure or substrate affinity of topo II enzymes isolated from these two cell lines. The suggestion that radiation protection by aminothiols is facilitated via endogenous cellular repair system (32-34), more specifically recombination-related repair processes (32-34), is consistent with the finding that WR-1065 can affect the activity of an enzyme involved in DNA topology such as topo II. Implications of the failure of WR-1065 exposure to protect or affect topo II activity in xrs-5 cells is at present unclear. However, these findings extend earlier observations

(32-34) that radiation protection by aminothiols is facilitated by the endogenous repair capacity of cells and that topo II is a nuclear enzyme which is affected by WR-1065.

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Table 1 *The effects of WR1065 and radiation on the activities of topo I and II in repair-proficient K1 and double-strand-break-deficient xrs-5 CHO cell lines, as determined by DNA relaxation and unknotting assays, respectively.*[a]

Cell Type	WR-1065	γ-ray	Topo I (Units/μg protein)[b]	Topo II (Units/μg protein)[b]
K1	–	–	112 ± 20	59 ± 14
K1	+	–	97 ± 28	26 ± 3[c]
K1	–	+	82 ± 22	53 ± 28
K1	+	+	96 ± 28	36 ± 13[d]
xrs-5	–	–	84 ± 16	53 ± 9
xrs-5	+	–	82 ± 20	48 ± 5
xrs-5	–	+	82 ± 22	49 ± 10
xrs-5	+	+	81 ± 24	47 ± 4

[a]Comparisons made to the corresponding untreated control groups using Student's 2-tailed T test. Comparisons not significant, $p \geq 0.386$, except as noted.

[b]Mean ± S.D. of four experiments.

[c]Significant difference at $p = 0.019$.

[d]Suggestive difference at $p = 0.061$.

Table 2 *The effects of WR1065 and radiation on the protein levels of topo II in repair-proficient K1 and double-strand-break-deficient xrs-5 CHO cell lines, as determined by immunoblotting using an anti-topo II specific antibody.*[a]

Cell Type	WR-1065	γ-ray	100 × Area[b]
K1	–	–	167 ± 55
K1	+	–	179 ± 49
K1	–	+	219 ± 21
K1	+	+	163 ± 39
xrs-5	–	–	162 ± 35
xrs-5	+	–	230 ± 35
xrs-5	–	+	171 ± 28
xrs-5	+	+	170 ± 30

[a]Comparisons made to the corresponding untreated control groups using Student's 2-tailed T test. All comparisons not significant, $p \geq 0.300$.

[b]Mean ± S.D. of at least three experiments.

FIGURE LEGENDS

Fig. 1. Survival curves for repair-proficient K1 and repair-deficient xrs-5 cell lines irradiated with 50-kVp x-rays. Cells were either treated with 4 mM of WR-1065 (■) or untreated (●). Experimental points represent the mean of three experiments; error bars represent the standard error of the mean. Survival curve parameters were determined by using a computer-fitted least-squares regression model.

Figure 2. Topo II (panel A) and topo I (panel B) activity in nuclear extracts from untreated and WR-1065-treated K1 cells. Nuclear extracts containing the following amounts of protein were assayed for topo-II-mediated unknotting and topo-I-mediated relaxing activities, as described in *Methods*: panel A, lane 1, 80 ng; lane 2, 40 ng; lane 3, 20 ng; lane 4, 10 ng; lane 5, 5 ng; panel B, lane 1, 100 ng; lane 2, 30 ng; lane 3, 10 ng; lane 4, 3 ng; lane 5, 1 ng; (–), no nuclear extract. This is a representative experiment. Data from four such experiments were used to determine the mean activities.

Fig. 3. Immunoblot analysis of topo II levels in nuclear extracts from untreated and WR-1065-treated K1 and xrs-5 cells. Logarithmically growing cells were washed twice by centrifugation at 1000 × *g* for 5 min in PBS-containing protease inhibitors and extracts. Nuclear proteins were subjected to gel electrophoresis through an 8% SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were incubated with anti-topo II antibody. The molecular weights shown on the right ordinate are those of topo II (MW 170,000) and its proteolytic products. Prestained standards with their molecular weights in thousands are shown on the left ordinate. Lanes 1-4, K1 cells; lanes 5-8, xrs-5 cells; untreated cells, lanes 1 and 5; WR-1065-treated but unirradiated cells, lanes 2 and 6; irradiated and treated with WR-1065, lanes 4 and 7.

Fig. 4. Immunoblot analysis of topo II levels in rapidly lysed cells. Conditions were similar to those described in Figure 3 with the exception that cells were lysed in electrophoresis sample buffer containing 2% SDS by boiling for 2 min. K1 (lane 1) and xrs-5 (lane 2) cell lysates.

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