WR-1065, an active metabolite of the cytoprotector amifostine, affects phosphorylation of topoisomerase II α leading to changes in enzyme activity and cell cycle progression in CHO AA8 cells

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Abstract. The effects of WR-1065 (2-((aminopropyl)amino)ethanethiol) on cell cycle progression, topoisomerase (topo) II α activity, and topo II α phosphorylation in Chinese hamster ovary (CHO) cells have been investigated. Exposure of CHO cells to 0.4 μ M of WR-1065 for 30 min did not effect cell cycle progression nor topo II α activity and phosphorylation status. However, concentrations ranging from 4 μ M to 4 mM were equally effective in significantly altering these three end points. Cell cycle progression was analysed by flow cytometry. Following a 30 min exposure to this range of concentrations, cells redistributed throughout the cell cycle with the most prominent changes being an accumulation of cells in G₂. Topo II α activity was measured using a kinetoplast DNA (kDNA) decatenation assay. Enzyme activity was reduced by 50% relative to control levels throughout the 4 μ M to 4 mM dose range tested. Likewise, topo II α phosphorylation levels, analysed using an immunoprecipitation assay and an antibody specific to the 170 kDa band of topo II, decreased between 42% to 48% of control levels. Inhibition of topo II α activity in cells exposed to WR-1065 is consistent with the associated observation of WR-1065 mediated cell cycle progression delay and build-up of cells in the G₂ phase of the cell cycle.

Aminothiol compounds have been a focus of radiation biology and oncology research because of their recognized radioprotective properties. Mechanisms of action attributed to these agents include the ability to scavenge free radicals, to take part in chemical repair via a hydrogen atom donation process, to induce intracellular hypoxia via auto-oxidation processes, and to effect intracellular enzymatic processes. With respect to enzymatic processes, it was reported as early as 1964 that aminothiols could inhibit DNA synthesis in murine bone marrow cells (LaSalle & Billen 1964). Since that time, aminothiol drugs have been reported to be capable of inhibiting DNA polymerase I activity in *Escherichia coli* (Billen 1983); affecting topoisomerase (topo) I activity in cell free extracts (Holwitt, Koda & Swenberg 1990); and inhibiting endonuclease activity in mammalian cells (Ramakrishnan & Catravas 1992).

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An aminothioli based radioprotector that has been the focus of many clinical studies is S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721, amifostine). This is a prodrug which requires dephosphorylation to its thiol form, 2-((aminopropyl)amino)ethane-thiol (WR-1065), by the enzyme alkaline phosphatase to be active as a cytoprotector. Exponentially growing mammalian cells exposed to millimolar amounts of this thiol under *in vitro* conditions can be effectively blocked in the G₂ phase of their cell cycle (Grdina *et al.* 1994). It has also been reported that WR-2721 can induce a similar response in bone marrow cells following its injection into Swiss albino mice (Devi, Prasanna & Rao 1992). This effect on cell cycle delay is transient. Under *in vitro* conditions, there is an immediate redistribution of cells throughout the cell cycle following the removal of the aminothioli from the culture medium (Sigdestad *et al.* 1988). A possible mechanism to account for this effect on cell cycle progression is the inhibition of activity by WR-1065, or its disulfide form, of an essential enzyme required for DNA synthesis. Candidate enzymes include the topoisomerases which are nuclear enzymes whose functions include the regulation of the topology of DNA during the processes of replication, transcription, chromatin condensation and segregation of chromosomes during cell division (Watt & Hickson 1994). Our laboratory was successful in demonstrating an inhibitory effect on topo II α activity following exposure of cells to a high dose of WR-1065, i.e. 4 mM (Grdina *et al.* 1994). The α -isoform of topo II is associated with newly replicating DNA (Nelson *et al.* 1986) and is a component of the nuclear matrix (Earnshaw & Heck 1985). Its expression and phosphorylation are highly regulated during the cell cycle, with the phosphorylation level being three- to 10-fold higher in the G₂/M phase (Heck *et al.* 1989) and its activity two- to threefold higher in G₂ than in earlier phases of the cell cycle (Sahyoun *et al.* 1986).

An exposure dose of 4 mM of WR-1065 to cells cannot be achieved under *in vivo* conditions due to toxicity limitations. Pharmacokinetic studies of the prodrug amifostine have been performed in which it was infused into cancer patients at a dose of 910 mg/m² (Shaw *et al.* 1994). Under these conditions the peak plasma concentration of WR-1065 was measured to be only 35 μ M. In this report we extend our study to include a broad range of doses of WR-1065, i.e. from 0.4 μ M to 4 mM. The effects of WR-1065 exposure on cell cycle progression, topo II α activity and phosphorylation using a CHO cell line are described.

MATERIALS AND METHODS

Cells and culture conditions

CHO AA8 cells were maintained as stock cultures in α -minimal essential medium (α -MEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (Sigma, St. Louis, MO), penicillin, and streptomycin (Gibco). The cells were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Drugs

The WR-1065 used in these studies was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, USA. Immediately prior to use, the drug was dissolved at a concentration of 1 M in phosphate-buffered saline (PBS, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl and 2.6 mM KCl, and filter-sterilized. Cells were exposed to final concentrations ranging from 0.4 μ M to 4 mM.

Preparation of nuclear extracts

Nuclear extracts were prepared from exponentially growing CHO AA8 cells following a method described in detail elsewhere (Grdina *et al.* 1994). Briefly, cells were treated with the appropriate concentration of WR-1065 or sham-treated for 30 min at 37°C with gentle shaking. The cells were then pelleted by centrifugation at 1000 rpm for 5 min at 4°C. Cell pellets were resuspended in 5 ml of ice-cold PBS containing protease inhibitors (20 μ g/ml aprotinin, 1 mM benzamidine, 50 μ g/ml leupeptin, 10 μ g/ml α 2-macroglobulin, 1 μ g/ml pepstatin, 1 mM phenylmethylsulfonylfluoride (PMSF), and 10 μ g/ml soybean trypsin inhibitor and incubated on ice for 15 min. The cells, about 2×10^7 , were pelleted by centrifugation and then homogenized. Nuclei were isolated by the method described in Tandou *et al.* (1984), and then nuclear proteins were extracted as described by Champoux & McConaughy (1976). Protein concentrations were determined using the Bradford method (Bradford 1976) and adjusted to 2 mg/ml by the addition of high-salt buffer (HSB) which consists of 20 mM Tris-HCl, pH 8.0, 5 mM KCl, 1 mM MgCl₂, 20 mM NaHSO₃ and 400 mM NaCl. An equal volume of HSB containing 60% glycerol was then added to the nuclear extracts, to give rise to a final protein concentration of 1 mg/ml.

Decatenating assay for the determination of topoisomerase II α activity

The enzymes for this assay were serially diluted nuclear extracts in a buffer of 30 mM NaPO₄, pH 7.0, 0.5 mM DTT, 50% glycerol and 0.5 mg/ml bovine serum albumin (BSA) in distilled water. The substrate was catenated kinetoplast DNA (kDNA) isolated from the mitochondria of *Crithidia fasciculata* (TopoGEN Inc., Columbus, OH). Reaction mixtures of 20 μ l contained 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 30 μ g/ml BSA, and 150 ng kDNA. The reactions were started by the addition of nuclear extract. After a 30-min incubation at 37°C, the reactions were terminated by the addition of 5 μ l of stop buffer (5% sarkosyl, 0.25% bromophenol blue and 25% glycerol). Samples were loaded on 1% agarose gels containing 0.5 μ g/ml ethidium bromide, electrophoresed at 26 V for 16 h in Tris/borate/EDTA (TBE) buffer, and photographed over an UV light source. Topo II α activity was quantitated by densitometric scanning of photographic negatives and levels of significance were determined using a two-tailed Student's *t*-test. Catenated kDNA remained in the well, while decatenated kDNA migrated as two bands, either nicked open circular kDNA or relaxed kDNA. One unit of decatenating activity is defined as the amount of enzyme that converts 50% of the substrate (catenated kDNA) into the reaction product (decatenated kDNA).

Immunoprecipitation of phosphorylated topoisomerase II α

CHO AA8 cells in exponential growth were washed twice in serum-free, phosphate-free RPMI 1640 medium (Gibco, Grand Island, NY) and then resuspended in 1 ml of this medium. After the addition of carrier-free (³²P)P_i (7.4 MBq/ml), the cells were incubated for 2 h at 37°C to allow for the incorporation of ³²P into ATP. Cells were pelleted and then resuspended into serum-free RPMI 1640 medium containing phosphate and WR-1065 at a concentration of 4 mM, 40 μ M, or 0.4 μ M. After incubation for 30 min at 37°C, the cells were pelleted and lysed in buffer containing 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.425 M NaCl, 0.05 M Tris-HCl (pH 7.4), and protease inhibitors. Lysates were adjusted with buffer to contain the same total amount of radioactivity as determined by measuring the resultant counts per minute in a scintillation counter. Aliquots (0.1 ml) were immunoprecipitated under conditions described in detail elsewhere (Constantinou *et al.* 1996). The anti-topo II α antibody was produced in rabbits by injecting the protein

product of the expression plasmid p56z11-1.8 provided by Dr Leroy Liu. The construction of the expression plasmid is described in detail elsewhere (Hwang *et al.* 1989). The specificity of the resultant antibody towards topo II α has been demonstrated by its ability to bind to a single band at 170 kDa (Grdina *et al.* 1994, Ganapathi *et al.* 1994). After pelleting and washing, the resultant complex was dissociated by boiling in 100 μ l of Laemmli buffer and electrophoresed in 8% SDS-polyacrylamide gels. The gels were stained, dried, and autoradiographed. Topo II α phosphorylation was quantitated by laser densitometric scanning of the 170 kDa band. Levels of significance were determined using a paired Student's *t*-test.

Flow cytometry

DNA content of cells was determined by flow cytometry as described in detail elsewhere (Grdina *et al.* 1994). At time zero, the appropriate concentration of WR-1065 was added to each plate, and the cells were incubated for 30 min at 37°C. The drug was removed by washing the cells twice with PBS, and fresh medium was added to the plates. At each point, plates were removed from the incubator, and the cells were trypsinized, washed twice with PBS, and centrifuged at 1000 rpm for 5 min at room temperature. Cells were then stained with 4',6-diamidino-2-phenylindole (Gohde, Schumann & Zante 1978). Flow cytometry patterns were obtained using a PARTEC PAS-II (Particle Analyzing System, Partec AG, Basel, Switzerland), and cell cycle components were identified by computer analysis (Phoenix Flow Systems Inc., San Diego, CA) using an algorithm based on the work of Dean & Jett (1974).

RESULTS

The standard *in vitro* exposure conditions used in assessing the cytoprotective properties of WR-1065 requires the presence of the drug during irradiation at concentrations above 1 mM (Grdina *et al.* 1995). Under these conditions WR-1065 inhibits both cell cycle progression and topo II α activity (Grdina *et al.* 1994). In this investigation CHO AA8 cells were exposed to concentrations of WR-1065 ranging from 0.4 μ M to 4 mM. Presented in Figure 1 are a series of flow cytometry DNA histograms which describe the effects of these concentrations of thiol on cell cycle progression. Cells were exposed to WR-1065 for 30 min and then were washed free of the drug. The result of this treatment was a build-up of cells in the G₂ phase of the cell cycle. To facilitate a comparison of the various treatment groups, the relative percentages of cells in the various phases of the cell cycle are presented in Figure 2. Each of the concentrations of WR-1065 evaluated was equally effective in causing a build-up of cells in G₂.

Topo II α activity was measured using a decatenation assay to detect the conversion of a catenated substrate (kDNA) to decatenated products (nicked open circular or relaxed kDNA) in the presence of ATP. Comparisons were made between untreated control CHO AA8 cells and cells exposed to WR-1065 for 30 min at concentrations ranging from 0.4 μ M to 4 mM. A 30-min exposure time was chosen to facilitate a comparison between topo II α activity and cell cycle progression data presented in Figures 1 and 2. A photograph of a representative gel is presented in Figure 3. The magnitude of inhibition of enzyme activity, i.e. approximately 50% (see Figure 4), was comparable for all concentrations of WR-1065 evaluated above 0.4 μ M. No effect on topo II α activity was observed at a concentration of 0.4 μ M.

Activation or inhibition of enzymes can be regulated by their phosphorylation status. Topo II α phosphorylation status was, therefore, examined using an immunoprecipitation

Figure 1. Representative DNA histograms obtained by flow cytometry describing the effects of a 30 min exposure of WR-1065 as a function of concentration on the subsequent distribution of cells throughout the cell cycle.

assay. A topo II α -specific antibody, which was obtained by injecting the protein product of an expression plasmid p56z11-1.8m into rabbits, was used. The resulting data are presented in Figure 5a,b. Densitometer scanning of the 170 kDa band in each gel representing topo II α was performed and the data are contained in Table 1 for comparison. Treatment of CHO AA8 cells with either 4 mM or 40 μ M WR-1065 for 30 min significantly reduced topo II α phosphorylation levels by $42\% \pm 5.2\%$ (SEM) and $48\% \pm 8.6\%$ compared to untreated

Figure 2. Percentage of cells distributed in the G₁, S and G₂ phases of the cell cycle as a result of computer analysis of DNA histograms presented in Figure 1 demonstrating the effect of WR-1065 as a function of concentration on cell cycle progression.

Figure 3. Topo II α activity in nuclear extracts isolated from control and cells exposed to 4 mM of WR-1065 for 30 min. Nuclear extracts containing the following amounts of protein were assayed for topo II α -mediated decatenation of kDNA: lane 1, no nuclear extract; lanes 2 and 8, 500 ng; lanes 3 and 9, 250 ng; lanes 4 and 10, 125 ng; lanes 5 and 11, 62.5 ng; lanes 6 and 12, 31.25 ng; lanes 7 and 13, 15.625 ng; lane 14, decatenated kDNA marker; lane 15, linear kDNA marker. This photograph represents a single representative gel, and data from three independent experiments were used to determine mean activities.

control cells ($P = 0.016$ and $P = 0.044$, respectively). Phosphorylation of topo II α was not significantly affected in cells exposed to $0.4 \mu\text{M}$ of WR-1065 which is consistent with the failure of this concentration of drug to affect the decatenation assay, i.e. the experimental system used to measure enzymatic activity.

DISCUSSION

Amifostine is a well characterized cytoprotective agent. Its active free thiol form, designated WR-1065, has been extensively investigated. Cells exposed to millimolar concentrations of WR-1065 experience a perturbation of cell cycle progression as evidenced by an accumulation of cells in G_2 phase (Sigdestad *et al.* 1988) and an inhibition of topo II α activity (Grdina *et al.* 1994). However, these studies have focused on relatively high doses of WR-1065 which cannot be achieved *in vivo* (Shaw *et al.* 1994). It was of interest, therefore, to characterize the effects of WR-1065 on cell cycle progression and topo II α enzyme activity at concentrations achievable under *in vivo* conditions.

As described in Figures 1 and 2, WR-1065 was equally effective at concentrations ranging from $4 \mu\text{M}$ to 4 mM in perturbing cell cycle progression. The change in cell cycle distribution was characterized by a build-up of cells in the G_2 phase of the cell cycle. No effect was observed for concentrations of WR-1065 below $4 \mu\text{M}$ (data not shown). Cell division is an important step in the fixation of DNA damage and its associated consequences of cell death, mutagenesis, and cellular transformation (Chu & Malling 1968, Farber 1984). It is not surprising, therefore, that WR-1065 which is known to protect against all of these end points would also be capable of affecting cell cycle progression. Presumably, the consequence of

inducing a transient delay and a build-up of cells in G₂ without concomitant cytotoxicity would be the allowance of relatively more time for repair processes to work, thus allowing for an enhanced magnitude and/or fidelity of repair which would be reflected in higher cell survival and a reduction in mutation frequency.

While there are a myriad of proteins that are known to be involved in processes relating to cell cycle progression, topo II α is one which has been well characterized and found to be required for successful DNA synthesis. This enzyme undergoes significant cell cycle dependent alterations in both amount and activity. The level of topo II α increases just prior to or at the onset of DNA synthesis and it continues to increase through S and G₂ phase,

Figure 4. The average percentage reduction of topo II α activity as a function of concentration of WR-1065 are plotted from three separate experiments. Error bars represent standard errors of the mean (SEM). All WR-1065 exposures were limited to 30 min.

(a)
Phosphorylated TopoII α

(b)
Phosphorylated TopoII α

Figure 5. Autograph of representative gels describing phosphorylated topo II α extracted from CHO AA8 cells. The samples were adjusted for equal cpm prior to immunoprecipitation with topo II α (170 kDa) specific antibody and separation by SDS-polyacrylamide gel electrophoresis. (a) Lane 1, untreated control; lane 2, cells exposed to 4 mM WR-1065 for 30 min. (b) Lane 1, untreated control cells; lane 2, cells treated with 40 μ M WR-1065 for 30 min; lane 3, cells treated with 0.4 μ M WR-1065 for 30 min. Data from three such sets of gels were used to calculate mean values of phosphorylation levels.

Table 1. Effects of WR-1065 on topoisomerase II phosphorylation levels^a

WR-1065 concentration	4 mM	4×10^{-2} mM	4×10^{-4} mM
–WR-1065	0.424 ± 0.122^b	0.440 ± 0.072	0.405 ± 0.090
+WR-1065	0.259 ± 0.117	0.250 ± 0.085	0.233 ± 0.058
<i>P</i> value ^c	0.016*	0.044*	0.108

^aMean topoisomerase II phosphorylation levels in CHO AA8 cells following a 30 min exposure to WR-1065.

^bAverage peak area (AU mm) of topoisomerase II bands as determined by laser densitometry $\pm$ the standard error of the mean.

^cDetermined using paired Student's *t*-test. Significant differences indicated by asterisk.

with a peak level occurring in late G₂ phase (Heck *et al.* 1988). Inhibition of topo II activity leads to a perturbation of cell cycle progression as evidenced by a build-up of cells in G₂ phase (Constantinou *et al.* 1996). As described in Figures 3 and 4, topo II α activity was inhibited by 50% over the same range of concentrations of WR-1065 that were found to be effective in blocking cells in G₂ (see Figures 1 and 2). These results demonstrate a strong correlation between WR-1065 mediated effects on topo II α enzyme activity and cell cycle progression. It is reasonable to expect that these effects will occur under *in vivo* conditions since standard clinical dosing with amifostine gives rise to micromolar levels of WR-1065 in the blood of patients soon after administration (Shaw *et al.* 1994).

As described in Figure 5, WR-1065 appears to affect topo II α activity by altering its phosphorylation status. Protein phosphorylation is a well recognized process which is involved in the regulation of enzyme activity (Cochet & Chambaz 1983). At present it is not clear just how WR-1065 can effect the phosphorylation state of proteins. WR-1065 is an aminothiols whose disulfide form closely resembles the polyamine spermine (Mitchell *et al.* 1995). Following the exposure of cells to WR-1065, the disulfide will form as a result of intracellular redox processes. The disulfide in turn appears to possess many properties in common with polyamines. In particular, it is equally effective as spermine in competing for: (1) cellular uptake via well characterized polyamine transporter systems (Mitchell *et al.* 1995); (2) binding sites on DNA in mammalian cell nuclei (Newton *et al.* 1996); and (3) packaging of DNA molecules into relatively condensed structures (Savoye *et al.* 1997). Polyamines in turn are known to be involved in affecting DNA structure and gene expression (Balasundaram & Tyagi 1991), protein phosphorylation status (Cochet & Chambaz 1983), and cell cycle progression (Brooks 1995). Spermine has also been reported to be effective in inhibiting topo II enzyme activity under certain conditions (Pommier, Kerrigan & Kohn 1989). While it is unclear at present as to the specific kinases and/or phosphatases that are affected by WR-1065, it is proposed that the inhibitory effect of this agent on topo II α enzyme activity is mediated through its ability to affect the phosphorylation state of the enzyme in a manner similar to that attributed to polyamines.

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