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Effects of thiols on topoisomerase-IIα activity and cell cycle progression

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Abstract. Thiol containing compounds exhibiting antioxidant properties are currently being evaluated for use in cytoprotection and chemoprevention. Many of these have also been found to be effective in inhibiting cell cycle progression and cellular proliferation. *N*-Acetyl-L-cysteine (L-NAC), along with its nonmetabolically active stereoisomer *N*-acetyl-D-cysteine (D-NAC), together with captopril and dithiothreitol (DTT) were investigated to assess their effects on cell cycle progression as determined by flow cytometry. Topoisomerase-IIa (topo-IIα) activity, an enzyme involved in DNA synthesis, was also monitored as a function of drug dose using a kinetoplast DNA (kDNA) decatenation assay. Chinese hamster ovary (CHO) AA8 cells were exposed to each thiol at concentrations ranging from 4 μM to 4 mM for a period of 3 h. Following the removal of the thiols, cell cultures were followed for an additional 5 h to assess changes in cell cycle progression. L-NAC, which also serves as a precursor for glutathione (GSH) synthesis, effectively inhibited topo-IIa activity by at least 50% at all concentrations tested. Associated with this reduction in enzyme activity was a sixfold increase in the relative number of cells accumulating in G_2 phase. D-NAC, which is unable to participate in GSH synthesis, was only half as effective as L-NAC at each concentration tested in inhibiting topo-IIa activity as well as perturbing cell progression through G_2. In comparison, captopril, an inhibitor of angiotensin converting enzyme (ACE), had little effect on the progression of cells into G_2 phase. In contrast to the repressive effects of L-NAC and D-NAC, it enhanced topo-IIa activity over control values by ≈20%. DTT, a well characterized thiol known to be capable of reducing disulphides in proteins, was observed to be relatively ineffective in either perturbing cell cycle progression or affecting topo-IIa activity. This suggests an involvement of a mechanism(s) in addition to thiol mediated affects on reduction/oxidation processes. The inhibitory effects of L-NAC and D-NAC on topo-IIa activity, in contrast to the other two thiols, may be due in part to the presence of amine groups which could allow for their participation in polyamine related processes. The difference in the magnitude of the effect exhibited by L-NAC, as compared to D-NAC, on the repression topo-IIa activity also suggests a role for GSH in this process. Inhibition

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of cellular progression and proliferation by thiols can therefore be mediated by diverse mechanisms which include both cycle-phase specific (i.e. L-NAC and D-NAC) and non cell cycle specific (i.e. captopril) processes.

INTRODUCTION

Numerous thiol containing compounds are the focus of clinical investigation for use in cytoprotection and chemoprevention. These agents, by virtue of their antioxidant properties, offer the potential of being effective anticarcinogenic drugs. One such agent, *N*-acetylcysteine (NAC) has been used clinically to protect against liver toxicity caused by acetaminophen (Corcoran & Wong 1986) and as an mycolytic agent (De Flora *et al.* 1985). It has recently been evaluated in clinical trials as a chemopreventive agent in both Europe and the United States (De Flora *et al.* 1995). L-NAC, however, can affect potential carcinogenic processes through both a direct, thiol mediated, scavenging of free radicals as well as through a modulation of enzymatically driven processes. The L-isomer form of NAC is a precursor of intracellular cysteine production and subsequent synthesis of the cellular thiol, glutathione (GSH) (De Flora *et al.* 1985). In contrast, the D-isomer of NAC is an excellent control for use in evaluating only the thiol mediated component of the chemopreventive effect since it is resistant to hydrolysis and cannot be deacetylated or used in the biosynthesis of GSH (De Flora *et al.* 1985).

Another clinically important antioxidant is the angiotensin-converting enzyme (ACE) inhibitor, captopril (D-3-mercapto-2-methylpropanoyl-L-proline). With its introduction in 1981, captopril has proven to be an effective medication in the treatment of essential hypertension (Materson & Preston 1994). Like NAC, this drug has a thiol group and is an effective radical scavenger. It can perturb the rate of DNA synthesis and proliferation in exposed cell populations (De Flora *et al.* 1985; Volpert *et al.* 1996; Benzie & Tomlinson 1998).

The mechanism by which these thiol containing compounds affects DNA synthesis and cellular proliferation is at present unclear. Recently it was reported that the active thiol form (i.e. *N*-(2-mercaptoethyl)-1,3-diaminopropane; WR-1065) of the clinically studied cytoprotector, amifostine, was found to be a potent inhibitor of cell cycle progression (Grdina *et al.* 1994; Murley, Kamath & Grdina 1997). Exposure of cells to WR-1065 resulted in an inhibition of cellular proliferation and a significant build up of cells in the G_2 phase of the cell cycle (Grdina *et al.* 1994). This phenomenon was linked to the drug's ability to inhibit the activity of an important enzyme required for DNA synthesis, i.e. topoisomerase-IIa (topo-IIα). While absolute protein levels were unaffected by exposure to a 4-mM dose of thiol, topo-IIa enzyme activity was reduced by over 50% as compared to control levels (Grdina *et al.* 1994). Using an immunoprecipitation assay with an antibody specific to topo-IIa it was demonstrated that levels of phosphorylated topo-IIa decreased to between 42% and 48% of control levels, suggesting a thiol mediated post-translational effect (Murley *et al.* 1997).

To assess whether thiol mediated effects on cellular progression and proliferation can be attributed to an inhibition of topo-IIa activity, four thiol containing compounds, two of which are currently used in the clinic, were evaluated using a kinetoplast DNA (kDNA) decatenation assay and flow cytometry. These included the metabolically active L-NAC, the inactive isomer D-NAC, the ACE inhibitor captopril, and, for comparative purposes, the reducing agent dithiothreitol (DTT).

MATERIALS AND METHODS

Cells and culture conditions

Chinese hamster ovary (CHO) AA8 cells were maintained as stock cultures in a-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% CO_2 and 95% air at 37°C.

Drugs

L-NAC, dithiothreitol and captopril were commercially obtained (Sigma, St. Louis, MO). D-NAC was synthesized according to the method described by Sheffner et al. (1966). All four drugs were stored at −20°C in a desiccator. Immediately prior to use, each drug was dissolved in phosphate buffered saline (PBS, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl and 2.6 mM KCl) and filter sterilized. Stock solutions were made up at 1 M, and the appropriate dilution was added directly to cell culture medium. No cytotoxic effects were observed for any of the drugs at the concentrations tested. Cells were exposed to each drug at concentrations ranging from 0.4 μM to 4 mM in order to facilitate a direct comparison between their effects on cell cycle progression and topo-IIa activity with that of WR1065 as described elsewhere (Murley *et al.* 1997).

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, aliquots of cells were treated with the appropriate concentration either of L-NAC, D-NAC, captopril or DTT for 3 h at 37°C with gentle shaking. Untreated controls were handled in the same manner. Following treatment, cells were pelleted by centrifugation at 1000 rpm for 5 min at 4°C and then resuspended in 5 ml ice-cold PBS containing protease inhibitors (20 μg/ml aprotinin, 1 mM benzamidine, 50 μg/ml leupeptin, 10 (g/ml α2-macroglobulin, 1 μg/ml pepstatin, 1 mM phenylmethylsulphonylfluoride, and 10 μg/ml soybean trypsin inhibitor) and incubated on ice for 15 min. Cells were then re-pelleted and subsequently homogenized, nuclei were isolated (Taudou *et al.* 1984), and nuclear proteins were then extracted (Champoux & McConaughy 1976). Protein concentrations were determined using the Bradford method (Bradford 1976) and then adjusted to 2 mg/ml by the addition of a high salt buffer (20 mM Tris-HCl, pH 8.0, 5 mM KCl, 1 mM $MgCl_2$, 20 mM $NaHSO_3$ and 400 mM NaCl). An equal volume of this buffer containing 60% glycerol was then added to the nuclear extracts to achieve a final protein concentration of 1 mg/ml.

Decatenating assay for the determination of topoisomerase-IIαa activity

The decatenating assay used to determine topo-IIa activity is described in detail elsewhere (Grdina *et al.* 1994; Murley *et al.* 1997). Nuclear extracts were serially diluted in a buffer of 30 mM $NaPO_4$, 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 (l contained 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 30 μg/ml BSA and 150 μg kDNA. The reactions were initiated with the addition of the nuclear extracts. After 30 min at 37°C the reactions were terminated by the addition of 5 μl of stop buffer (5% sarkosyl, 0.25% bromophenol blue and 25% glycerol). Samples were

loaded on 1% agarose gels containing 0.5 μg/ml ethidium bromide, electrophoresed at 26 V for 16 h in Tris/borate/EDTA buffer, and photographed over a UV light source. Topo-IIa activity was quantified by densitometric scanning of photographic negatives and significance levels were determined using a two-tailed Student's *t*-test. Catenated kDNA remained in the well, while the 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).

Flow cytometry

DNA content was measured by flow cytometry (Murley *et al.* 1997). At time zero, the appropriate concentration of a selected thiol compound was added to each cell culture plate. Cells were incubated for 3 h at 37°C. Each drug tested was removed by washing the cells twice with PBS before the addition of fresh medium. Cell cultures were then incubated at 37°C for selected time intervals and then removed. Single cell suspensions were made with trypsin, and were then washed twice with PBS and pelleted. Cells were then stained with 4,6-diamidino-2-phenylindole (DAPI) (Gohde *et al.* 1979). Flow cytometry patterns were obtained using a PARTEC PAS-II (Particle Analysing 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

For comparative purposes, a control DNA histogram representing untreated cells is presented in Figure 1. Presented in Figure 2 are flow cytometry generated DNA-histograms describing the effects of L-NAC, D-NAC, captopril, and DTT on cell cycle progression. For purposes of comparison, the standard treatment condition consisted of exposing exponentially growing cells to a nontoxic and cytoprotective dose of 4 mM of each thiol for a 3-h incubation period. Following drug removal, cell populations were followed for an additional 5 h to assess changes in cell cycle distribution. An untreated control population was monitored for 6 h by flow cytometry to evaluate changes in cell cycle distribution as a function of time (see Figure 3). No significant changes in cell cycle distributions were observed. Treatment with L-NAC, however, resulted in a large build-up of cells in the G_2 phase of the cell cycle. While less in magnitude, similar changes in cell cycle distribution were also observed following exposure of cells to D-NAC (see Figure 4). Neither captopril nor DTT, however, was as effective as L-or D-NAC in perturbing cell cycle progression (see Figure 4).

In an earlier report it was demonstrated that the free thiol form of amifostine (i.e. WR-1065) could both inhibit topo-IIa enzyme activity and perturb cell cycle progression as evidenced by an accumulation of treated cells in G_2 phase (Murley *et al.* 1997). It was of interest therefore to assess and contrast the ability of each of these thiol containing compounds to affect topo-IIa activity. Topo-IIa activity was measured using a decatenation assay to detect the conversion of a catenated substrate (kDNA) to decatenated products such as nicked open circular or relaxed DNA strands in the presence of ATP. Comparisons were made between untreated exponentially growing control cells and cell populations exposed to L-NAC, D-NAC, captopril, and DTT. Cells were exposed for 3 h at 37°C to a drug concentration of 4 mM. An exposure time of 3 h was chosen to facilitate a comparison between

changes in topo-IIa activities and the cell cycle progression data presented in Figures 2 and 4. Representative photographs of gels describing the effects of 4 mM concentrations of each of the thiol containing compounds on topo-IIa activity are presented in Figures 5, 6, 7 and 8 for L-NAC, D-NAC, captopril and DTT, respectively. Each of the resulting bands formed in the gels was quantified by densitometric scanning and the data from three replicate experiments representing four thiol doses are presented for comparison in Table 1. L-NAC was the most effective in inhibiting topo-IIa activity. D-NAC was also effective in inhibiting topo-IIa activity, but the magnitude was only about half that observed for L-NAC. DTT was evaluated and found to have no significant effect on either cell cycle progression or topo-IIa activity.

Effects of L-NAC, D-NAC and captopril on topo-IIa activity were also evaluated at concentrations ranging from 4 μM to 0.4 mM (see Fig. 7). While the greatest effect for each of these drugs was observed at a concentration of 4 mM, their respective abilities to affect topo-IIa activity was relatively uniform throughout the concentration range tested.

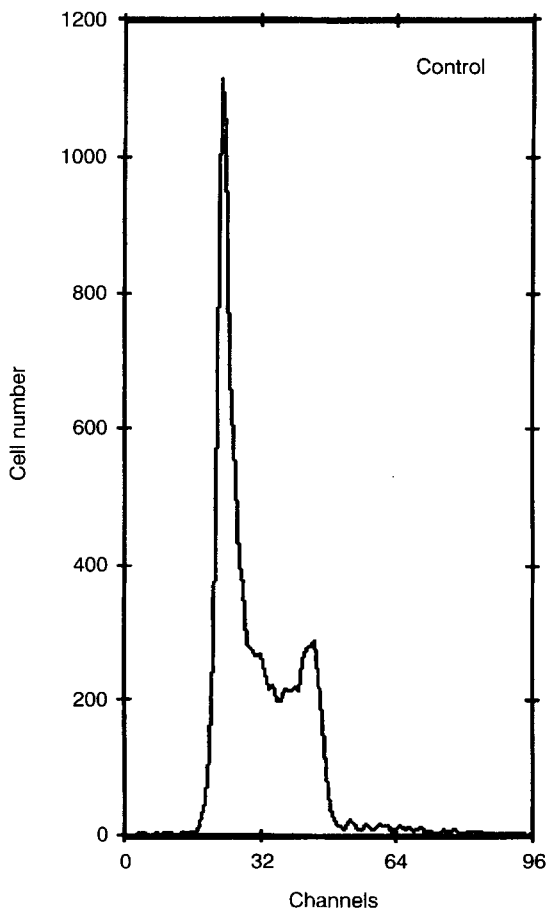

Figure 1. Representativer DNA histograms obtained by flow cytometry describing the cell cycle distribution of control cells prior to treatment.

DISCUSSION

Thiol containing drugs having antioxidant properties are considered to be excellent candidates for use in the chemoprevention of cancer. In addition to their ability to scavenge free radicals and thus reduce oxidative damage, many of these drugs are also precursors for the synthesis of endogenous cellular protectants such as GSH (De Flora *et al.* 1985), and can act as specific inducers of phase II enzymes (Hong & Sporn 1997) and modifiers of apoptotic processes (Held *et al.* 1996; Hong & Sporn 1997). Furthermore, selected thiols can also inhibit DNA synthesis and cellular proliferation. The present study was prompted by an earlier report in which the active free thiol of amifosine (i.e. WR-1065), a drug effective in inhibiting mutagenic (Grdina *et al.* 1992; Kataoka *et al.* 1992), transforming (Hill *et al.* 1986; Balcer-Kubiczek *et al.* 1993) and carcinogenic (Milas *et al.* 1984; Carnes & Grdina 1992) processes, was found to inhibit cellular progression and topo-IIa enzyme activity (Murley *et al.* 1997). Specifically, a 30-min exposure of cells to WR-1065 at concentrations ranging from 4 μM to 4 mM all resulted in a 50% inhibition of topo-IIa enzyme activity and a fourfold build-up of cells in G_2 phase of the cell cycle. This effect was not demonstrable at a drug

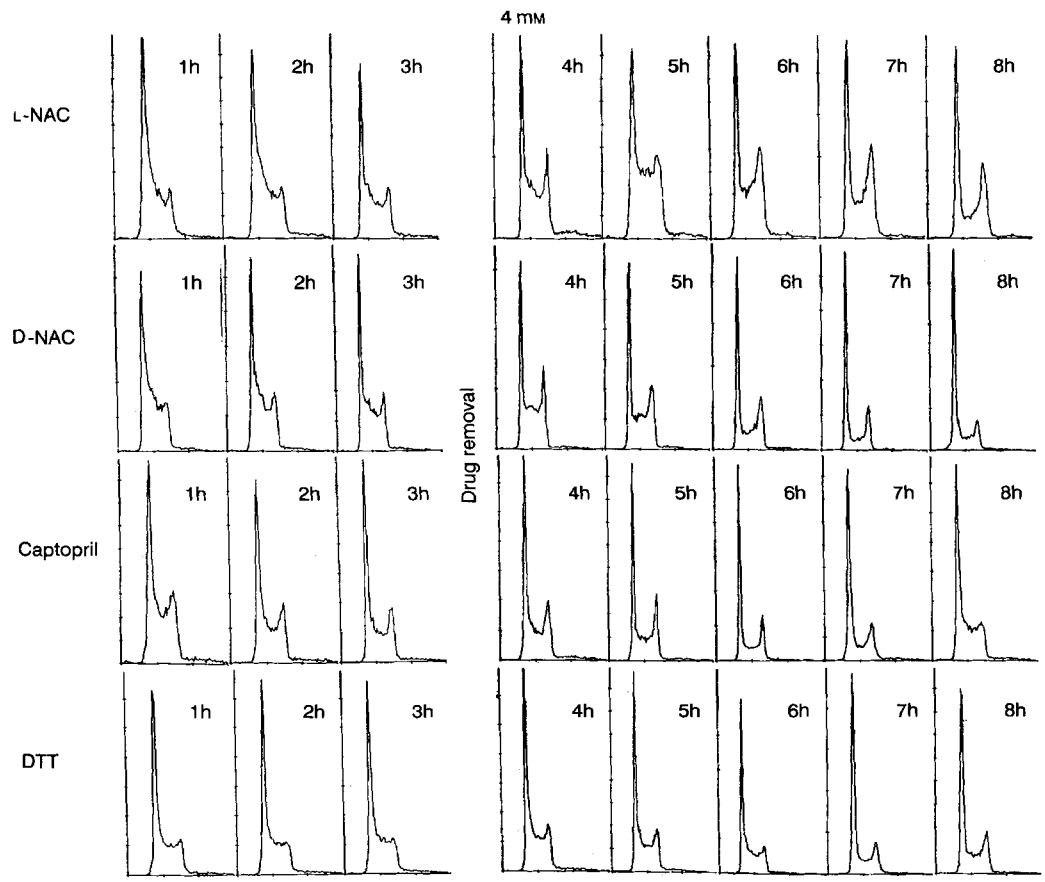

Figure 2. Representative DNA histograms obtained by flow cytometry describing the effects of a 4-mM concentration of L-NAC, D-NAC, captopril, or DTT as a function of time on the subsequent distribution of cells throughout the cell cycle.

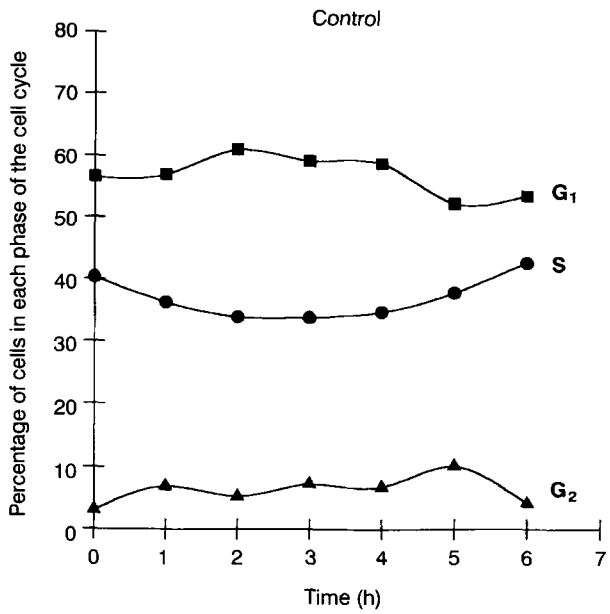

Figure 3. Percentage of untreated control cells distributed in the G_1 (■), S (●), and G_2 (▲) phases of the cell cycle as a function of time.

concentration of 400 nM (Murley *et al.* 1997). To determine whether the topo-IIa effect observed for WR-1065 treated cells is a phenomenon which can be mediated by thiols in general, we extended our investigation to include the thiols L-NAC, D-NAC, captopril and DTT.

L-NAC was chosen for study because it has been extensively evaluated for use as a chemopreventive agent (Corcoran & Wong 1986; Bongers *et al.* 1995; De Flora *et al.* 1995). In addition to its free radical scavenging properties, it can be deacetylated by *N*-acetylases to form L-cysteine and eventually the intracellular thiol GSH (De Flora *et al.* 1985). Its stereoisomer D-NAC, in contrast, is resistant to enzymatic deacetylation and cannot support GSH biosynthesis. Both isomers of NAC have been reported to be capable of suppressing DNA synthesis and cellular proliferation suggesting that metabolism of L-NAC to L-cysteine and GSH is not a requirement for the effect (Ferrari *et al.* 1995). However, as shown in Figures 2 and 4, while both isomers of NAC can perturb cell cycle progression, the L-form is more effective than the D-form. This is presumably due to an enhanced level of GSH induced by L-NAC metabolism. Consistent with data reported for WR-1065 (Grdina *et al.* 1994; Murley *et al.* 1997), both of these aminothiols can inhibit topo-IIa enzyme activity as described by a decatenation assay (see Figs 3 and 4) and promote, under *in vitro* conditions, the cellular uptake of cysteine and subsequent increase of glutathione biosynthesis (Issels & Nagele1989). That the stereoisomer L-NAC was more effective than D-NAC in inhibiting topo-IIa activity suggests a potential role for GSH in this process. Also consistent with this possibility is data derived using dexamethasone. Cells exposed to dexamethasone exhibited both increased levels of glutathione (Millar *et al.* 1983) and reduced levels of topo-IIa activity (Goodlad & Clark 1994).

Neither DTT nor captopril was as effective as the two stereoisomers of NAC in altering cell cycle distribution (Figures 1 and 2) and repressing topo-IIa enzyme activity (Figures 5 and 6 and Table 1) in exposed cells. DTT, because of its low redox potential, is capable of maintaining monothiols in a reduced state and of reducing disulphides in a quantitative manner. It is considered to be a model thiol with which to study thiol mediated processes (Held *et al.* 1996). Its relative poor ability to affect topo-IIa activity and subsequent cell cycle progression suggests the involvement of factors in addition to those related only to radical scavenging and redox mediated signalling. In contrast to NAC and WR-1065, DTT is not an aminothiol. The presence of amine groups may have an important role to play in facilitating changes to both topo-IIa enzyme activity and subsequent cell cycle progression by thiol containing compounds.

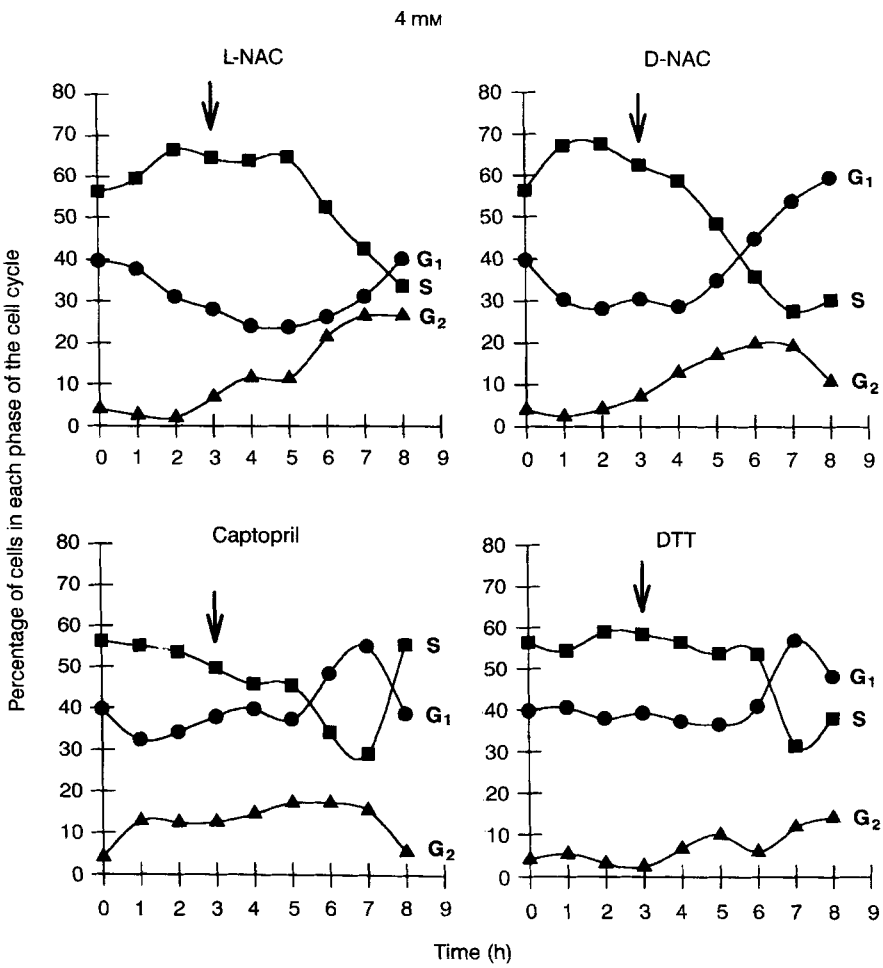

Figure 4. Percentage of cells distributed in the G_1 (●), S (■), and G_2 (▲) phases of the cell cycle as a result of the computer analysis of DNA histograms presented in Figure 2 demonstrating the effects of L-NAC, D-NAC, captopril and DTT exposures, as a function of time, on cell cycle distributions.

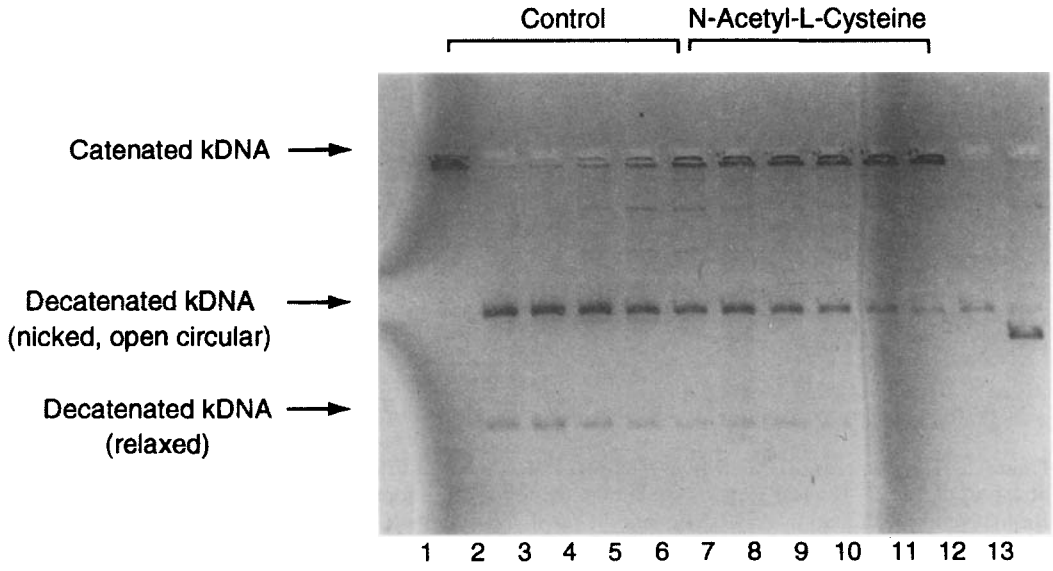

Figure 5. Topoisomerase-IIα activity in nuclear extracts isolated from control and cells exposed to 4 mM of L-NAC for 3 h. Nuclear extracts containing the following amounts of protein were assayed for topo-IIα-mediated decatenation of kinetoplast DNA (kDNA): lane 1, no nuclear extract; lanes 2 & 7, 500 ηg; lanes 3 & 8, 250 ηg; lanes 5 and 10, 62.5 ηg; lane 12, decatenated kDNA marker; and lane 13, linear kDNA marker. This photograph represents a single representative gel, and data from three independent experiments were used to determine mean activities.

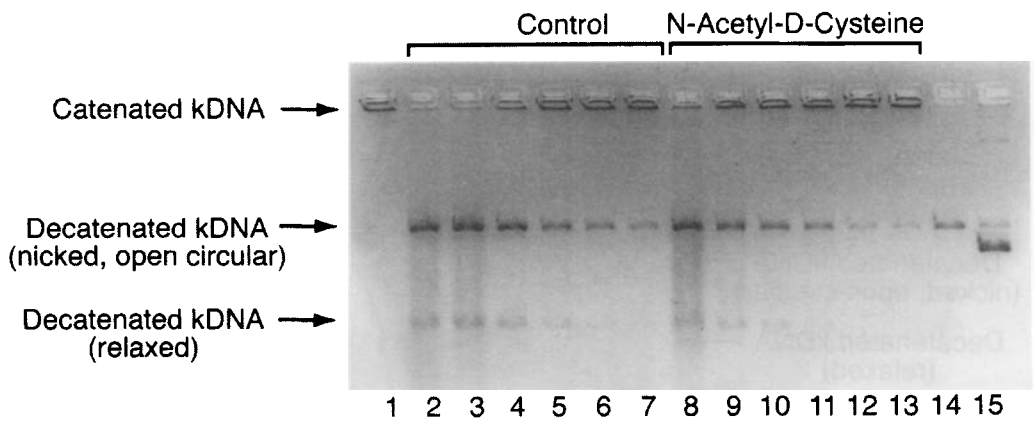

Figure 6. Topoisomerase-IIα activity in nuclear extracts isolated from control and cells exposed to 4 mM of D-NAC for 3 h. Nuclear extracts containing the following amounts of protein were assayed for topo-IIα-mediated decatenation of kDNA: lane 1, no nuclear extract; lanes 2 & 8, 500 ηg; lanes 3 & 9, 250 ηg; lanes 4 & 10, 125 ηg; lanes 5 & 11, 62.5 ηg; lanes 6 & 12, 31.25 ηg; lanes 7 & 13, 15.625 ηg; lane 14, decatenated kDNA marker; and lane 15, linear kDNA marker.

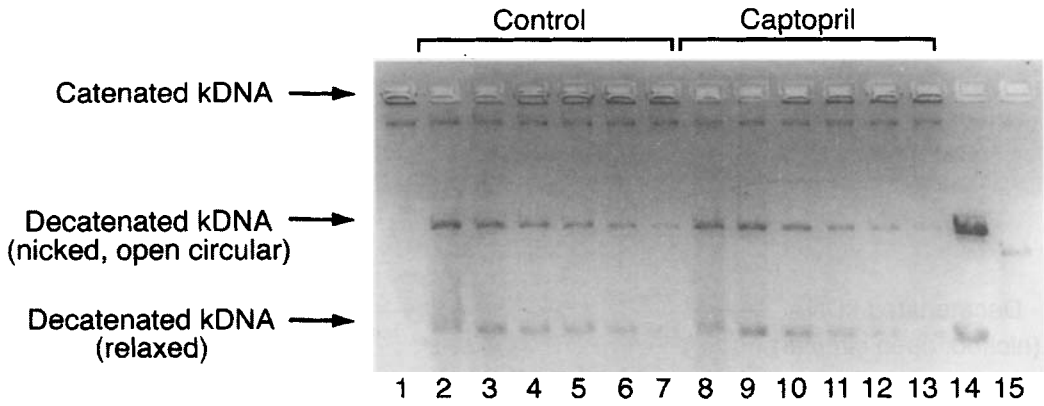

Figure 7. Topoisomerase-IIα activity in nuclear extracts isolated from control and cells exposed to 4 mM of captopril for 3 h. Nuclear extracts containing the following amounts of protein were assayed for topo-IIα-mediated decatenation of kDNA: lane 1, no nuclear extract; lanes 2 & 8, 500 ηg; lanes 3 & 9, 250 ηg; lanes 4 & 10, 125 ηg; lanes 5 & 11, 62.5 ηg; lanes 6 & 12, 31.25 ηg; lanes 7 & 13, 15.625 ηg; lane 14, decatenated kDNA marker; and lane 15, linear kDNA marker.

Captopril is also not an aminothiol. In contrast to NAC and WR-1065, it was found to stimulate topo-IIa activity but without a significant concomitant effect on perturbing cellular progression (Figures 1, 2 and 5 and Table 1). The stimulatory effect of captopril on topo-IIa activity extended over the same concentration range (i.e. 4 μM to 4 mM) as was observed for the inhibitory effects of L-and D-NAC (see Figure 7) and WR-1065 (Murley *et al.* 1997). Captopril is known to be an inhibitor of cellular proliferation in a number of mammalian cell systems including human lung fibroblasts (Nguyen *et al.* 1994) and human mammary ductal carcinoma cells (Small *et al.* 1997). Consistent with our results, the inhibition of cellular proliferation was not accompanied by significant changes in the cell cycle distribution as determined by flow cytometry. Rather, the effect was attributed to a general antimetabolic effect exerted by captopril (Small *et al.* 1997).

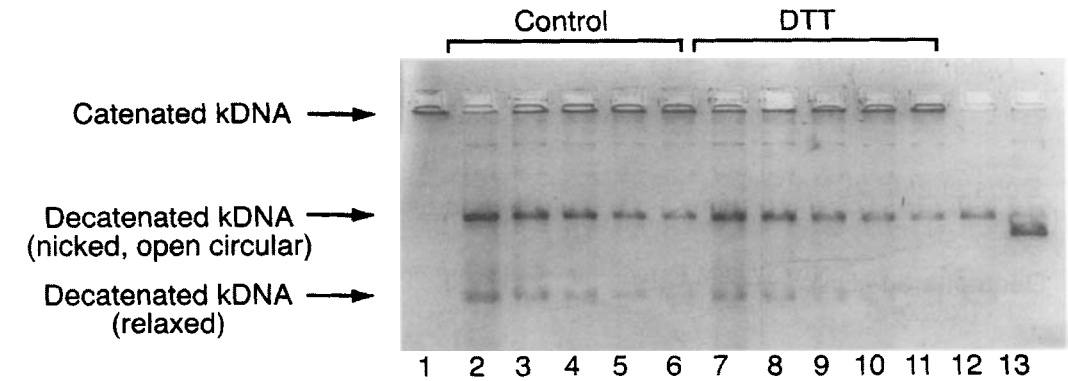

Figure 8. Topoisomerase-IIα activity in nuclear extracts isolated from control and cells exposed to 4 mM of DTT for 3 h. Nuclear extracts containing the following amounts of protein were assayed for topo-IIα-mediated decatenation of kDNA: lane 1, no nuclear extract; lanes 2 & 7, 500 ηg; lanes 3 & 8, 250 ηg; lanes 4 & 9, 125 ηg; lanes 5 & 10, 62.5 ηg; lanes 6 & 11, 31.25 ηg; lane 12, decatenated kDNA marker; and lane 13, linear kDNA marker.

Table 1. Relative effects of thiols on topoisomerase IIα activity[a]

Drug	Concentration	Activity[b]	*P*-value[c]
L-NAC	0	100%	
	4 mM	25% ± 10%	<0.001*
	0.4 mM	54% ± 5%	<0.001*
	0.04 mM	49% ± 14%	0.007*
	0.004 mM	39% ± 6%	<0.001*
D-NAC	0	100%	
	4 mM	44% ± 4%	<0.001*
	0.4 mM	91% ± 9%	0.286
	0.04 mM	76% ± 2%	<0.001*
	0.004 mM	71% ± 3%	<0.001*
Captopril	0	100%	
	4 mM	139% ± 4%	<0.001*
	0.4 mM	104% ± 4%	0.178
	0.04 mM	124% ± 14%	0.047*
	0.004 mM	124%[d]	

[a]Topoisomerase-IIα activities were calculated after normalizing untreated cells to a value of 100%.
[b]Mean topoisomerase-IIα activity in drug-treated cells determined from three separate experiments ± the standard error of the mean.
[c]Determined using a two-tailed Student's *t*-test. Significant differences indicated by an asterisk.
[d]Only one experiment was performed, but the data are included to show the consistency of the observations.

While considerable interest exists in the evaluation of thiols for use in chemoprevention, no single mechanism of action can adequately describe their effects on cellular proliferation. It is known that oxidation of thiols can generate hydrogen peroxide in a metal catalysed reaction (Held *et al.* 1996) which can then stimulate oxidation sensitive transcription factors and effect gene expression (Zheng *et al.* 1998). It is unclear at present as to the precise role this mechanism plays in thiol mediated changes in cell cycle progression. Unlike both NAC and WR-1065, captopril can inhibit cellular proliferation through a mechanism not involving the repression of topo-IIa activity. In contrast, DTT, at the concentrations tested, had very little effect, if any, on either topo-IIa activity or cellular progression even though it is considered to be capable of reducing disulphides in proteins (Held *et al.* 1996). The differences in the effects between the thiols investigated may be due in part to the presence or absence of amine groups. In over 4400 thiol containing compounds tested by the US Army Medical Research and Development Command, the aminothiol class of thiols (i.e. NAC and WR-1065) was recognized as superior to other classes tested for use in cytoprotection (Sweeney 1979). The most effective compounds identified had a free or potentially free thiol group separated by two or three carbons from an amine group. It may be that selected aminothiols such as NAC and WR-1065 can participate not only in redox reactions but also in intracellular polyamine mediated reactions that result in modifications of enzyme activity. For example, the polyamine spermine has been reported to significantly modulate topo-IIa activity in mammalian cells (Pommier *et al.* 1989). Both NAC and WR-1065 may participate in a similar mechanism to affect the activity of this enzyme.

Thiol containing compounds offer significant promise for the development of useful and effective agents for chemopreventive applications. Their effects on processes leading to changes in cellular proliferation can be very diverse. A better understanding of the underlying mechanisms of action as they relate not only to redox related signalling but also

to specific transcriptional, translational, and post-translational processes will facilitate the design and development of a highly specific acting class of agents for use in chemoprevention.

ACKNOWLEDGEMENTS

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