0026-895X/04/6603-627–634$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 66:627–634, 2004

Vol. 66, No. 3
3220/1171138
Printed in U.S.A.

DNA Topoisomerase I As One of the Cellular Targets of Certain Tyrphostin Derivatives

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Received January 16, 2004; accepted June 4, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

DNA topoisomerases (topo) are the cellular targets of several anticancer drugs used today in the clinic. Our previous work demonstrated that certain tyrphostin derivatives, known as protein tyrosine kinase antagonists, are catalytic inhibitors of DNA topoisomerases I (topo I) in vitro. In this study, we examined the ability of tyrphostin derivatives to affect the activity of topo I in the cell (in vivo) and determined their in vivo mode of action. Two approaches were used; in the first, we examined the direct effect of the treatment of tumor cells with tyrphostins on the activity, level, and post-translational modifications of the cellular topo I. The second approach was to determine the influence of pretreatment of tumor cells with tyrphostin on the cellular induced effects of camptothecin (CPT), a known inhibitor of topo I. The results show that treatment of fibrosarcoma cells with tyrphostin inhibited the DNA relaxation activity of topo I but did not reduce the level of topo I protein. Tyrphostin treatments caused conformational changes of the cellular topo I probably by binding to the enzyme. Pretreatment of the cells with tyrphostin before CPT prevented the CPT-induced degradation of topo I and reduced the enzyme-DNA cleavable complexes and the ubiquitination/sumoylation of the enzyme. These data suggest that topo I is one of the cellular targets of tyrphostin and that this drug acts in vivo (in the cell) as a catalytic inhibitor of the enzyme that alters the binding of the enzyme to the DNA.

Mammalian DNA topoisomerases are the targets of several anticancer drugs used today in the clinic (Wang, 1994; Wang et al., 1997; Pommier, 1998; Pommier et al., 1999; Li and Liu, 2000). Topoisomerases are classified as type I or type II, and members of each family of enzymes are distinct in sequence, structure and function (Roca, 1995; Wang, 1996; Champoux, 2001). Type IB topoisomerase (topo IB) plays important role in DNA replication, RNA transcription, DNA recombination, chromosome condensation and the maintenance of genomic stability (Champoux, 2001; Wang, 2002). Topo IB can relax both positive and negative supercoiled DNA by the formation of a transient single-strand DNA break in which the active site tyrosine becomes attached to the 3′-phosphate end of the cleaved strand followed by the rotation of the DNA and religation process (Champoux, 2001; Wang, 2002). The topo I inhibitor camptothecin (CPT) is a potent anticancer agent and its derivatives topotecan and irinotecan are used today for the treatment of ovarian cancer and colorectal cancer, respectively (Mathijssen et al., 2002). In our previous work, we showed that certain tyrphostin derivatives, known as protein tyrosine kinase antagonists (Levitzki, 1992; Levitzki and Gazit, 1995), inhibited the DNA relaxation activity of purified calf thymus topo I in vitro (Aflalo et al., 1994; Argaman et al., 2003). These compounds, unlike CPT, altered the binding of topo I to the DNA probably by interacting with topo I protein and affecting its processivity (Aflalo et al., 1994; Argaman et al., 2003). The ability to inhibit topo I is not a general characteristic of all tyrphostin derivatives because compounds such as AG-17 and AG-213 did not have a significant inhibitory effect on topo I activity. In addition, those tyrphostins that inhibited topo I activity did not affect the activity of other DNA binding proteins such as DNA polymerase I, DNA ligase, and virus reverse transcriptase (Aflalo et al., 1994). However, although the in vitro study suggested that certain tyrphostins may represent a new

This work was supported by research grants received from the chief scientist of the Israeli Ministry of Health, the Israel Cancer Association, The Morants T contribution for cancer research, and the Kaplutshnik Faiga contribution for cancer research.

ABBREVIATIONS: topo, DNA topoisomerases; topo I, DNA topoisomerase type I; CPT, camptothecin; AG-17, (3,5-di-*t*-butyl-4-hydroxybenzylidene)-malononitrile; AG-213, 3,4-dihydroxy-α-cyanothiocinnam-amide; AG-1387, α-cyano-(3,4-dihydroxy)-5-iodo-*N*-(3-phenylpropyl)cinnamide; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PAGE, polyacrylamide gel electrophoresis; AG-555, 2-cyano-3-(3,4-dihydroxy-phenyl)-*N*-(3-phenyl-propyl)-acrylamide; AG-1387, 2-cyano-3-(3,4-dihydroxy-5-iodo-phenyl)-*N*-(3-phenyl-propyl)-acrylamide; AG-1386, 2-cyano-3-(4-hydroxy-3-iodo-5-methoxy-phenyl)-*N*-(3-phenyl-propyl)-acrylamide; AG-552, 2-cyano-*N*-(3-phenyl-propyl)-acetamide; AG-974, (3,4-dihydroxy 5-iodo benzylidene)malononitrile; AG-18, (3,4-dihydroxy benzylidene)malononitrile; AG-1714, (4-nitro benzylidene)malononitrile.

group of topo I inhibitors, it is still unclear whether topo I is one of the cellular targets of tyrphostins. In this study, we used two approaches to investigate this possibility: 1) examination of the effect of treatment of tumor cells with tyrphostins on the activity, protein level, and expression of the cellular topo I, and 2) the influence of tyrphostin treatment on the CPT-induced effects in tumor cells, namely stabilization of the enzyme-DNA cleavable complexes (Wang, 1994, 1996, 2002; Wang et al., 1997; Pommier, 1998; Pommier et al., 1999), Ubiquitination/sumoylation of topo I protein (Desai et al., 1997, 2001), and topo I degradation (Desai et al., 1997, 2001, 2003). We show that treatment of cells with tyrphostin AG-1387 inhibited the DNA relaxation activity of topo I in a dose- and time-dependent manner. This inhibition was caused not by the reduction in topo I protein level but by possible conformational changes in topo I protein. In addition, pretreatment of tumor cells with tyrphostins reduced the level of CPT-induced cleavable complexes of topo I-DNA and prevented the CPT-induced degradation of topo I.

Materials and Methods

Compounds. Stock solutions of the different tyrphostin derivatives, 10 mM in 100% DMSO, were stored at −70°C and diluted in dimethyl sulfoxide before being added to the reaction mixture or to the cell culture medium. Stock solutions of CPT (Sigma-Aldrich) at 10 mM in dimethyl sulfoxide were stored in aliquots at −70°C.

Enzyme. Purified calf thymus topoisomerase I was purchase from MBI Fermentas (Hanover, MD).

Cells. Murine fibrosarcoma tumor cell variants IC9 and IE7 (De Baetselier et al., 1980) and immortalized murine NIH/3T3 cell lines were grown in Dulbecco's minimal essential medium containing 5% penicillin, 5% glutamine, and 10% fetal calf serum.

Cell Cytotoxicity. Cells (10^4/well) were exposed to increasing concentrations of tyrphostins for 48 h. Cell viability was determined by the Neutral Red cytotoxicity assay (Babich and Borenfreund, 1990), and the CC_{50} (concentration of the drug that caused 50% of cell death) was calculated.

Preparation of Nuclear Extracts. Nuclear extracts from the different cell lines were prepared as described previously (Auer et al., 1982; Sambrook et al., 1989) except that a mixture of protease inhibitors (final concentrations, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml antipain, and 100 μg/ml phenylmethylsulfonyl fluoride) were added to the extraction buffers. Total protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) .

Topo I Assay. Purified calf thymus topo I (5 units) or increasing concentrations of nuclear proteins were added to a topo I reaction mixture containing, at a final volume of 25 μl, 20 mM Tris-HCl, pH 8.1, 1 mM dithiothreitol, 20 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 30 μg/ml bovine serum albumin. and 225 ng of pUC19 supercoiled DNA plasmid (MBI Fermentas, Hanover, MD). Different concentrations (10–750 μM) of the various tyrphostin derivatives were added. After incubation at 37°C for 30 min, the reaction was terminated by adding 5 μl of stopping buffer (final concentration, 1% SDS, 15% glycerol, 0.5% bromphenol blue, and 50 mM EDTA, pH 8). The reaction products were analyzed by electrophoresis on 1% agarose gel using a Tris-borate/EDTA buffer (89 mM Tris-HCl, 89 mM boric acid, and 62 mM EDTA) at 1 V/cm, stained by ethidium bromide (1 μg/ml), and photographed using a short-wavelength UV lamp (ChemiImager 5500; Alpha Innotech, San Leandro, CA).

Densitometric analysis of the results were performed using the AlphaEasFC image processing and analysis software, and the percentage of topo I inhibition was calculated using the following equation: [1 − (sample/control)] × 100.

Identification of Topo I mRNA. Total RNA was prepared from tyrphostin-treated or untreated cells (10^7/flask) by the Tri Reagents kit (MRC Inc., Cincinnati, OH). Northern blot analysis was performed using radiolabeled T1B plasmid (received from Dr. Y. Pommier, National Institutes of Health, Bethesda, MD) as a probe.

Determination of the Level of Topo I Protein by Immunoprecipitation and Western Blot Analysis. Equal amounts of nuclear proteins derived from IC9 cells that were subjected to different treatments, were analyzed by Western blot analysis as described previously (Sambrook et al., 1989; Kaufmann and Svingen, 1999) using either anti-topo I antibodies (Genosys, Cambridge, UK), anti-topo II antibodies (Genosys), anti-poly(ADP-ribose) polymerase antibodies (BIOMOL Research Laboratories, Plymouth Meeting, PA), or anti-β-actin antibodies (MP Biomedicals, Irvine, CA). The immunocomplexes were detected by enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoprecipitation of topo I protein from nondenatured nuclear protein extracts derived from IC9 cells that were subjected to different treatments was performed as follows: anti-topo I antibodies were added to 100 μg of nuclear proteins (1:100 dilution) for overnight incubation at 4°C on a rotating wheel. Fifty microliters of preswelled protein A-Sepharose beads (0.1 g/ml of Tris-EDTA buffer) were added and the sample was incubated for 1 h. at 4°C. The immunocomplexes were harvested by centrifugation at 10,000*g* for 2 min and washed 5 times with Tris-EDTA buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA) and analyzed by Western blot analysis using anti-topo I antibodies.

Band Depletion Assay. The band depletion assay was performed essentially as described previously (Kaufmann and Svingen, 1999). IC9 cells (10^7/flask) were treated with CPT (60 μM) for 45 min, with tyrphostin AG-1387 (240 μM) for 3 h, or were preincubated with 240 μM tyrphostin for 3 h, followed by CPT treatment (60 μM) for an additional 45 min. The cells were removed from the flask by scraping without removing the medium (to prevent reversal of the cleavable complex), followed by centrifugation (1000 rpm, 5 min, 4°C). Denaturing buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, and 1 mM EDTA) was immediately added to the cell pellet, and the samples were mixed by vortexing until the turbidity disappeared. Samples were sonicated to diminish viscosity (40 bursts of 2 min each at two-thirds of the maximum output of microtip). Equal volumes of protein samples were analyzed on 10% SDS-PAGE followed by Western blot analysis with anti-topo I antibodies.

Detection of Topo I-Ub/SUMO-1 Conjugates. The detection of topo-I conjugates was performed as described previously (Desai et al., 1997).

IC9 cells (10^7/flask) from the different drug treatments were removed from the flask by a scraper without removing the medium. After centrifugation, the cell pellet was lysed using an ice-cold lysis buffer (0.2 N NaOH and 2 mM EDTA). Cell lysate was sonicated and neutralized with 1/10 volume of 2 N HCl. Topo I conjugation buffer (10% Nonidet P-40, 1 M Tris-HCl, pH 7.4, 0.1 M $MgCl_2$, 0.1 M $CaCl_2$, 10 mM dithiothreitol, and 1 mM EGTA) was added at 1/10 volume followed by the addition of a mixture of protease inhibitors (100 μg/ml each of leupeptin, pepstatin, antipain, aprotinin, and phenylmethylsulfonyl fluoride). P1 nuclease (200 μg/ml) was added and the samples were incubated for 2 h at 37°C. SDS-PAGE was performed with 5% polyacrylamide gels, and Western blotting was performed using anti-topo I antibodies.

Results

Identification of the Tyrphostin Derivatives that Exhibited the Most Potent Inhibitory Effect on Purified Topo I in Vitro. In addition to our previous study that demonstrated the efficacy of certain tyrphostin derivatives as anti-topo I inhibitors (Aflalo et al., 1994; Argaman et al., 2003), we further examined other tyrphostin derivatives (de-

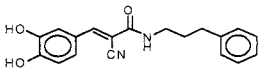
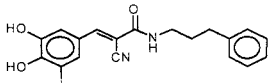
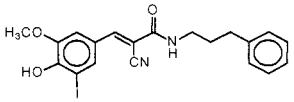
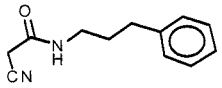
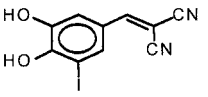
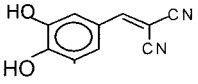
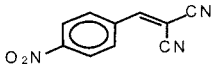
scribed in Table 1) to identify the most potent tyrphostin derivative that inhibits topo I. Increasing concentrations of tyrphostins were added to the topo I-specific reaction mixture and their effect on the DNA relaxation activity of purified calf thymus topo I was examined. The IC_{50} values for each compound were calculated as described previously (Aflalo et al., 1994). The results summarized in Table 1 show that the examined tyrphostins differed in their capacity to inhibit topo I. Tyrphostin AG-1387 inhibited 50% of topo I activity at 60 μM, whereas more than 10-fold AG-17 or AG- 1714 (750 μM) were needed to inhibit 50% of topo I activity. Moreover, the results in Table 1 indicate that the alkyl side chain attached to the cinnamonitrile of a specific tyrphostin is necessary for topo I inhibition but the alkyl side chain alone (AG-552) was not effective.

Inhibition of Topo I Activity in Nuclear Extracts Derived from Different Tumor and Immortalized Cell Lines. From the tyrphostins tested above, we chose AG-1387, AG-1386, and AG-1714 (which exhibited the most potent, moderate, and lowest topo I inhibitions, respectively) for the examination of their effects on topo I activity in nuclear cell extracts. Nuclear protein lysate were prepared from a murine fibrosarcoma tumor cell line (designated IC9) as described previously (Aflalo et al., 1994). Equivalent amounts of nuclear proteins were added to the topo I reaction mixture in the absence or presence of increasing doses of tyrphostins. The results were subjected to densitometric analysis, and the IC_{50} for each compound was calculated (see *Materials and Methods*). The results depicted in Table 2 show that among the tested tyrphostin derivatives, AG-1387 exhibited the most potent inhibitory effect on topo I activity in nuclear extracts derived from IC9 cells, whereas AG-1714 did not affect topo I activity. Similar results were obtained when nuclear protein extracts derived from another fibrosarcoma cell line (IE7) or immortalized NIH/3T3 cell lines were examined (data not shown). These results are compatible with the aforementioned data that were obtained with purified enzyme. The effect of these compounds on the growth of fibrosarcoma cell lines was examined and the results revealed that the CC_{50} (cytotoxic concentrations that caused 50% cell death after 48 h of treatment) for AG-1387, AG-1386, AG-974, and AG-1714 were 50, 100, 250, and 350, respectively (Table 2).

TABLE 1

Inhibition of purified topo I by various tyrphostin derivatives

Purified calf thymus topo I (1 unit) was added to a specific topo I reaction mixture in the presence of increasing doses of tyrphostins. The reaction products were analyzed using agarose gel electrophoresis, and the percentage of inhibition was calculated using densitometric analysis. The results are means ± S.D. of five different experiments.

Tyrphostin	Structure	Inhibition of Purified Topo I (IC_{50})
		μM
AG-555		100 ± 15
AG-1387		60 ± 9
AG-1386		100 ± 13
AG-552		No effect
AG-974		750 ± 110
AG-18		750 ± 130
AG-1714		1000 ± 150

Inhibition of Topo I DNA Relaxation Activity in Tyrphostin-Treated Cells. The results described above together with our previous work suggest that certain tyrphostin derivatives inhibited topo I activity when purified enzyme or nuclear extracts were used. To investigate the ability of these compounds to affect the activity of topo I in the cell, we first treated the fibrosarcoma tumor cell line IC9 with different concentrations of AG-1387 for various incubation periods. Nuclear extracts were prepared, and increasing nuclear protein concentrations were added to a topo I reaction mixture. The results depicted in Fig. 1A show that the level of inhibition of topo I in the cell was dose- and time-dependent. A significant decrease in topo I activity (60 ± 10%) was observed in nuclear extracts derived from IC9 cells treated with 240 μM AG-1387 for 2 h. (Fig. 1, A and B). However, lower concentrations (60 or 120 μM) or shorter (0.5 or 1 h) periods of tyrphostin treatment reduced topo I activity only slightly (10–15%) (Fig. 1A). Longer periods of tyrphostin treatment (6 and 24 h) result in a significant reduction in the level of topo I inhibition. The effect of this compound on topo I reduced inhibition to 25% in 6 h (Fig. 1A) and to 10% in 24 h (data not shown) after the addition of the drug to the cell culture.

The decrease in topo I activity in AG-1387 treated cells could be caused by 1) a transient decrease in the level of topo I protein, 2) post-translational modifications of topo I protein, or 3) A direct inhibition of the catalytic topo I activity by tyrphostin as suggested by our previous work (Aflalo et al., 1994). We investigated each of these possibilities using (in most of the experiments) the dose of tyrphostin (240 μM) that exhibited the most effective inhibition of topo I in the cell.

Tyrphostin Treatment Did not Affect the Expression and Level of Topo I. To examine the effect of treatment of

TABLE 2

Inhibition of topo I derived from nuclear extracts of murine fibrosarcoma (IC9) tumor cell lines, by tyrphostin derivatives.

For cell cytotoxicity determination, IC9 cells were treated with increasing doses of tyrphostins for 48 h, and cell cytotoxicity was determined by Neutral Red cytotoxicity assay. Topo I activity in the nuclear extracts was measured in the absence or presence of increasing doses of tyrphostins. The results are means ± S.D. of five different experiments. Densitometric analysis was performed, and the IC_{50} was calculated.

Tyrphostin	Cell Cytotoxicity (CC_{50})	Inhibition of Topo I Activity (IC_{50})
	μM	
AG-1387	50 ± 5	80 ± 3
AG-1386	100 ± 12	105 ± 6
AG-1714	350 ± 33	No effect

cells with tyrphostin AG-1387 on the expression of topo I, the IC9 fibrosarcoma cells were treated with AG-1387 for various incubation periods. The cellular RNA was extracted and subjected to Northern blot analysis using a radiolabeled topo I probe (T1B plasmid; received from Dr. Y. Pommier). The results depicted in Fig. 2A show that tyrphostin treatment did not affect the level or the size of topo I mRNA.

The effect of tyrphostin treatment on the level of topo I protein was examined by Western blot analysis using specific anti-topo I antibodies. The results depicted in Fig. 2B show that tyrphostin treatment did not affect the level of topo I protein. Similar results were obtained when IC9 cells were treated with higher (300 μM) or lower (60 μM) doses of tyrphostin AG-1387 for various treatment periods (data not shown). These results suggest that the decrease in topo I activity observed after tyrphostin treatment was not caused by a decrease in the level of topo I protein.

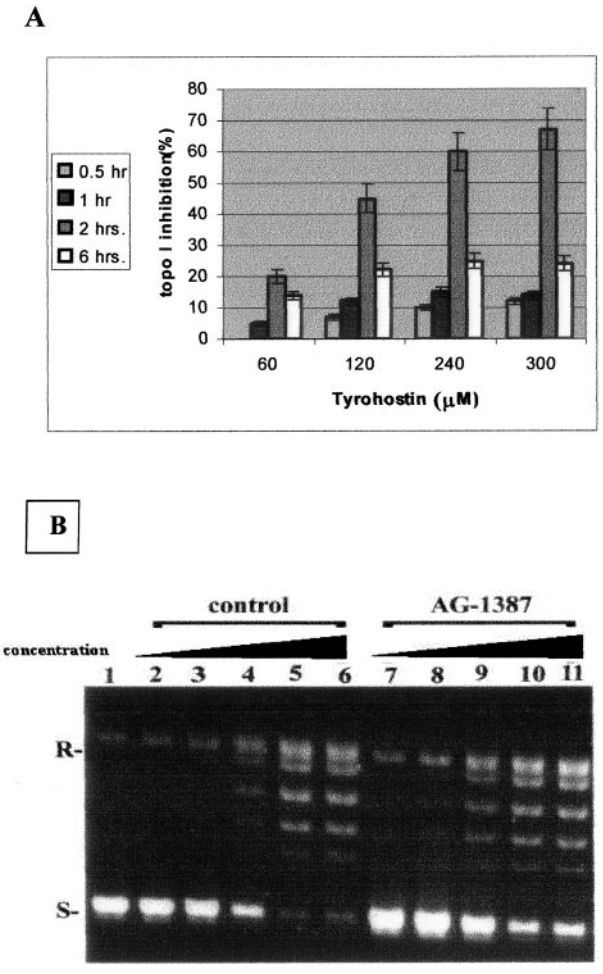


Fig. 1. Treatments of IC9 cells with increasing concentrations of tyrphostin AG-1387 decreased the activity of the cellular topo I. A, fibrosarcoma IC9 Cells (106 per flask) were treated, for different incubation periods, with increasing doses of AG-1387. Nuclear extracts were prepared from treated and control untreated cells, and increasing amounts of nuclear proteins (0.5, 1, 2, 4, and 6 ng) were added to a topo I reaction mixture. The reaction products were analyzed by agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed using a short wavelength UV lamp. Densitometric analysis was performed, and the percentage of topo I activity was calculated as described under *Materials and Methods*. The results are means ± S.D. of three different experiments. B, a representative agarose gel electrophoresis analysis of the topo I reaction products, obtained with increasing amounts (0.5, 1, 2, 4, and 6 ng) of nuclear extract proteins, derived from IC9 cells, untreated (lanes 2–6) and treated (lanes 7–11) with 240 μM AG-1387 for 2 h. Lane 1, control, supercoiled pUC19 DNA. R and S are the relaxed and supercoiled forms of the pUC19 DNA plasmid, respectively.

Tyrphostin Treatment Induced Conformational Changes in Topo I Protein. Topo I protein was immunoprecipitated by specific anti-topo I antibodies from the nonedenatured nuclear protein extracts, the immunocomplexes were subjected to Western blot analysis using the anti-topo I antibodies, and the level of the immunoprecipitated topo I was calculated. The results depicted in Fig. 3 show a significant decrease (65%) in the level of the immunoprecipitated topo I protein derived from the cells treated with tyrphostin for 2 h. Slighter effect (25%–10%) on the level of the immunoprecipitated topo I was observed after longer treatments (A, lanes 3–6 and 8, and B). These results suggest that a fraction of the topo I protein, derived from the tyrphostin-treated cells, was not recognized by the anti-topo I antibodies. This might be a consequence of conformational changes of the enzyme, which occurs after the treatment with tyrphostin. To confirm this assumption, purified topo I was mixed with tyrphostin AG-1387 and the enzyme was immunoprecipitated as described above. Western blot analysis with anti-topo I antibodies show a significant decrease (92%) in the level of the immunoprecipitated topo I when this enzyme was mixed with tyrphostin (Fig. 3C, top, compare lane 2 with lane 1). Tyrphostin treatment did not affect the immunoprecipitation of other proteins such as β-actin (Fig. 3C, bottom), suggesting that topo I protein undergoes conformational changes in the presence of tyrphostin AG-1387.

Tyrphostin Treatment Prevents CPT-Induced Topo I Degradation. The aforementioned results suggest that treatment of cells with tyrphostin cause a transient inhibition of topo I activity and conformational changes in topo I protein. Our previous results demonstrate that certain tyrphostin derivatives inhibited topo I activity by decreasing the ability of this enzyme to bind DNA in vitro (Aflalo et al., 1994). If tyrphostin treatment influences the DNA binding capacity of topo I in the cells, it might decrease or prevent the CPT-induced effects on topo I in CPT-treated cells. CPT acts by stabilizing the enzyme-DNA covalent complex, and it was previously shown that treatment of cells with CPT results in

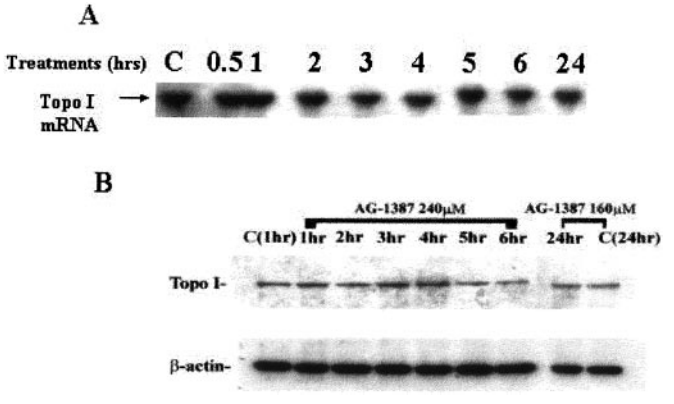


Fig. 2. Tyrphostin treatment of IC9 cells did not influence the level of topo I mRNA or protein. IC9 (10^7 per flask) cells were treated with tyrphostin AG-1387 for various intervals. The total cellular RNA was prepared, and Northern blot analysis was performed using radiolabeled T1B plasmid as a probe (A). Nuclear proteins (40 μg) were analyzed on SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti-topo I or anti-β- actin antibodies (B).

a significant reduction in the level and the activity of topo I. This reduction in the level of topo I is caused by a degradation of the topo I protein by the 20 S proteasome pathway and is not the consequence of a reduction in de novo synthesis (Desai et al., 1997, 2001, 2003).

To determine the effect of tyrphostin on the CPT-induced degradation of topo I, IC9 cells were preincubated with tyrphostin AG-1387 for 3 h followed by CPT treatment for additional 3 h. The results depicted in Fig. 4, A and D, show a significant decrease (82%) in the level of topo I protein in CPT-treated cells (compare lane 2 and column 2 with lane 1 and column 1, respectively) and no effect in tyrphostin-treated cells (lane 4 and column 4). Preincubation with tyrphostin followed by CPT treatment prevented (85%) the decrease in the level of topo I protein (compare lane 3 and column 3 with lane 2 and column 2, respectively). The above-described treatments did not influence the level of topo II protein (Fig. 4B) or β-actin (Fig. 4C). In addition, topo I activity was examined in nuclear extracts derived from the aforementioned treatments; the results depicted in Fig. 4E show a significant decrease in topo I activity after CPT treatment (compare lanes 6–9 with lanes 2–5), which was prevented by pretreatment with tyrphostin (compare lanes 14–17 to lanes 6–9). These results suggest that pretreatment of cells with tyrphostin specifically prevented the CPT-induced degradation of topo I.

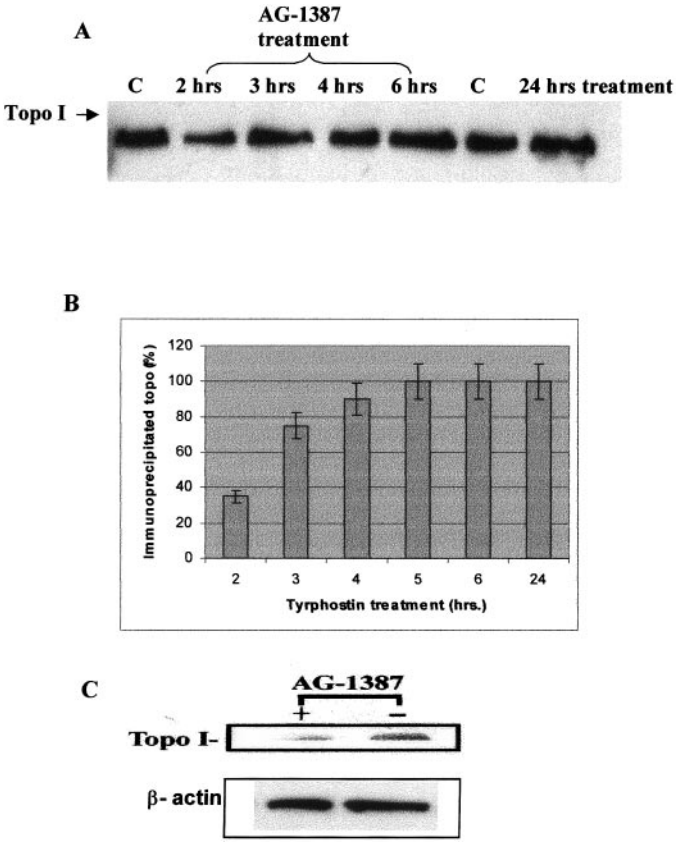


Fig. 3. Tyrphostin treatments influence the level of the immunoprecipitated topo I. A, IC9 Cells (10^6 per flask) were treated with 240 μM AG-1387 for various intervals. The topo I protein was immunoprecipitated from 100 μg of nuclear proteins using anti-topo I antibodies (1:100). The immunocomplexes were analyzed by SDS-PAGE followed by Western blot analysis with anti-topo I antibodies. B, densitometric analysis was performed and the percentage of the immunoprecipitated topo I was calculated. The results are means ± S.D. from three different experiments. C, AG-1387 (240 μM) was added to a buffer containing 20 units of purified topo I (top) or 40 μg of protein extract (bottom). Immunoprecipitation of the purified topo I or β-actin was performed from tyrphostin-treated or untreated samples by anti-topo I or anti-β-actin antibodies. The immunocomplexes were analyzed as described in A.

Because the degradation of topo I is mediated by the proteasome pathway, it is possible that tyrphostin treatment prevent the CPT-induced degradation of this protein by affecting the proteasome activity. To examine this possibility we used an in vitro proteasome activity assay described by Dick et al. (1997). IC9 cells were incubated with MG-132 (a proteasome inhibitor) or with tyrphostin AG-1387 for 3 h. Cellular protein extracts were prepared, and equal amounts of proteins were added to a proteasome reaction mixture containing a specific substrate (succinyl-Leu-Leu-Val-Tyr 7-amino-4-methylcoumarin); 20 S proteasome activity was determined by the hydrolysis of the substrate resulting in the liberation of fluorescence 7-amino-4-methylcoumarin, which was monitored by a fluorometer. Treatment of cells with MG-132 or tyrphostin resulted in 97% or 20% inhibition of the proteasome activity, respectively, suggesting that tyrphostin treatment might slightly influence the activity of the 20 S proteasome.

Pretreatment of IC9 Cells with Tyrphostin Reduced the Level of CPT-Induced Topo I-DNA Cleavable Complexes. In a transient reaction intermediate (termed the cleavable complex), topo I links covalently to DNA through tyrosine, leaving a DNA break with a free 5′-hydroxyl end. Treatment of cells with CPT result in the stabilization of these cleavable complexes and the prevention of the ligation step (Wang, 1994, 2002; Wang et al., 1997; Pommier, 1998). To examine the effect of pretreatment of cells with tyrphostin on the formation of CPT-induced topo I-DNA cleavable complexes, we used the "band depletion" assay (Kaufmann and Svingen, 1999). This assay is based on the observation that an increase in the amount of topo I bound to the DNA because of CPT treatment will cause a depletion in the level of free topo I protein. IC9 cells were preincubated with AG-1387 for 3 h, followed by CPT treatment for 45 min. Cells were immediately lysed with SDS, and the level of topo I protein was examined by Western blot analysis using specific anti-topo I antibodies. The results depicted in Fig. 5, A and E, demonstrate a significant decrease (90%) in the level of free topo I in CPT-treated cells (compare lane 2 and column 2 with lane 1 and column 1, respectively) and no effect on the level of topo I in cells treated with tyrphostin alone (compare lane 4 and column 4 with lane 1 and column 1, respectively). However, pretreatment with tyrphostin before CPT significantly prevented (75%) the CPT-induced depletion of topo I (compare lane 3 and column 3 to lane 1 and 2 and columns 1 and 2, respectively). Under these conditions, no significant changes in the levels of topo II (Fig. 5B), poly-ADP ribose polymerase (Fig. 5C), and β-actin (Fig. 5D) were observed.

Pretreatment of Cells with Tyrphostin Reduced the CPT-Induced Topo I-Ubiquitin/SUMO-1 Conjugates. It was shown previously that after CPT treatment, the topo I cleavable complex undergoes ubiquitination/sumoylation (Desai et al., 1997, 2001). To determine the effect of pretreatment with tyrphostin on this processes, IC9 cells were pretreated with tyrphostin AG-1387 for 3 h followed by CPT treatment for 10 min. Cell extracts were prepared as described previously (Desai et al., 1997, 2001) and equal vol-

umes were loaded on 5% polyacrylamide gel. Western blot analysis using anti-topo I antibodies was performed. The results depicted in Fig. 6 show the formation of topo I-UB/SUMO-1 conjugates in CPT-treated cells (lane 2). Pretreatment with tyrphostin reduced the level of these conjugates (lane 4), and treatment with tyrphostin alone did not cause topo I-Ub/SUMO-1 conjugates (lane 3).

Discussion

Tyrphostins are synthetic compounds specifically designed to inhibit the activity of protein tyrosine kinases. They have been examined in many biological systems and are successful antiproliferative agents (Levitzki, 1992; Levitzki and Gazit, 1995). In our previous work, we showed that certain tyrphostin derivatives inhibited the DNA relaxation activity of purified and unpurified (from nuclear extracts) topo I in vitro. Examination of the mode of action of these compounds, revealed that they inhibit the topo I activity by preventing the binding of this enzyme to the DNA (Aflalo et al., 1994). In addition, we showed that tyrphostin derivatives did not affect the activity of other DNA binding enzymes (i.e., DNA polymerase, DNA ligase, and reverse transcriptase), suggesting that the inhibition of topo I by these drugs is specific (Aflalo et al., 1994) To identify the chemical structure of tyrphostin that exhibited the most potent anti-topo I activity, we examined the effect of various tyrphostin derivatives on the DNA relaxation activity of purified and nuclear extract derived topo I. Tyrphostin AG-1387 exhibited the most potent inhibitory effect on both purified and unpurified enzyme and the data presented in Table 1 indicate that the alkyl side chain attached to the cinnamonitrile of a specific tyrphostin is necessary for topo I inhibition, but the alkyl side chain by itself (AG-552) was not effective. In addition, among the tested tyrphostins, AG-1387 exhibited the highest cytotoxic effect on the fibrosarcoma tumor cells (IC9 and IE7) compared with the other tested tyrphostins. These data suggest a direct correlation between the cytotoxic ability of certain tyrphostin derivatives and their capacity to inhibit topo I.

The ability of AG-1387 to inhibit topo I activity in vitro does not necessarily indicate that topo I is indeed the target of this drug in the cell. Therefore, in this research, we attempted to examine this possibility using two approaches. The first was to determine the effect of treatments of fibrosarcoma cells with different doses of AG-1387, at various intervals, on the activity, level, and modifications of the cellular topo I. A short incubation period (2 h) of the cells, with this drug, decreased by 60% the cellular DNA relaxation activity of topo I. The decrease in the enzyme activity was not caused by a reduction in the level of the topo I protein or by post-translational modifications. In addition, tyrphostin treatment did not induce the formation of topo I-Ub/SUMO conjugates. However, significant decreases in the level of the immunoprecipitated topo I (65%) caused by anti-topo I antibodies was observed in the tyrphostin-treated cells. These results suggest that AG-1387 caused conformational changes in the topo I protein that altered its recognition by the anti-topo I antibodies. These conformational changes might be caused by a direct interaction of AG-1387 with topo I protein or by modification of the enzyme proteins as a result of the other effect of tyrphostins (i.e., inhibition of protein tyrosine kinases). The possible direct interaction of tyrphostin with topo I protein was confirmed by an in vitro assay in which purified topo I was mixed with the drug followed by immunoprecipitation assay. A significant decrease (92%) in the level of the immunoprecipitated topo I in the presence of tyrphostin was observed. These data are compatible with our previous in vitro study in which we suggested that tyrphostins probably binds topo I protein, altered its conforma-

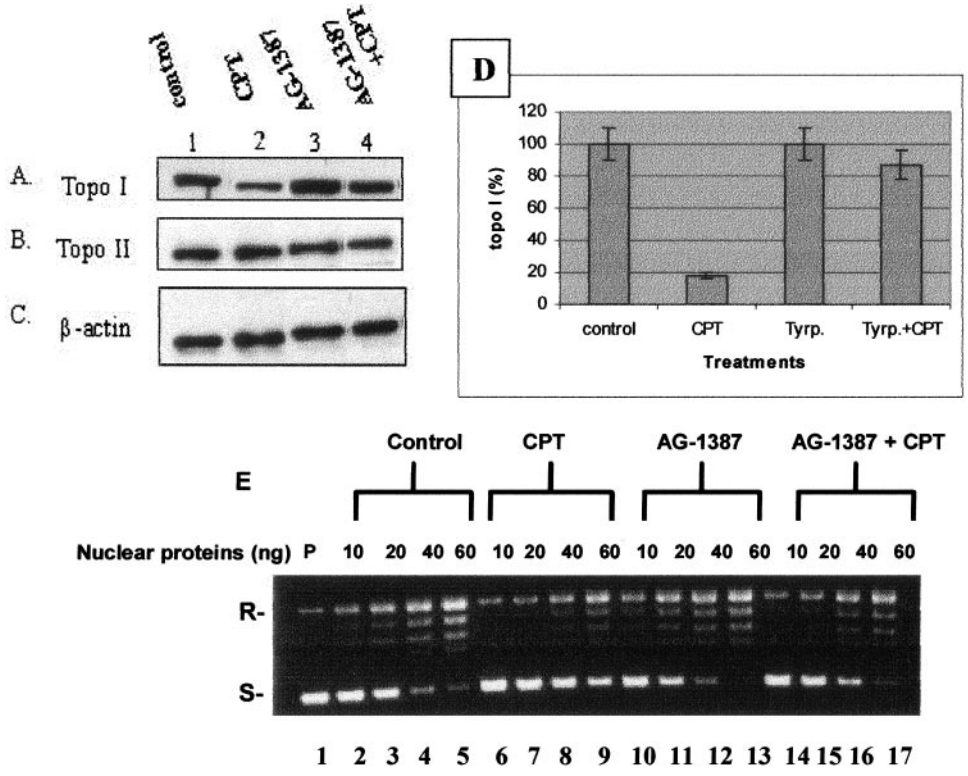
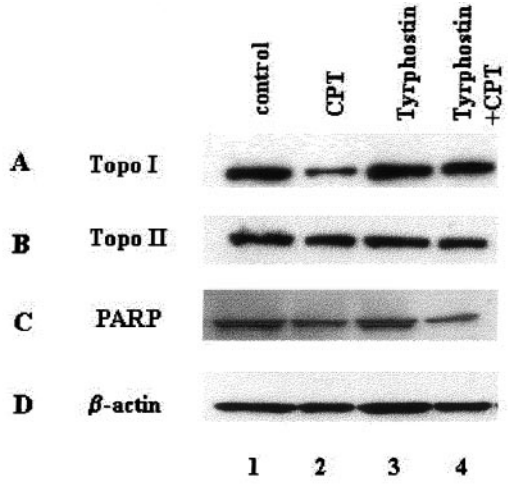


Fig. 4. Pretreatment of IC9 cells with tyrphostin reduced the CPT-induced degradation of topo I. IC9 cells were treated with CPT (60 μM) for 3 h (lane 2), AG-1387 (240 μM) for 6 h (lane 3), or AG-1387 for 3 h followed by treatment with CPT for 3 h (lane 4); results from control untreated cells are shown in lane 1. Nuclear extracts were prepared and equivalent amounts of nuclear proteins were analyzed on SDS-PAGE followed by Western blot analysis with anti-topo I antibodies (A), anti-topo II antibodies (B), or anti-β-actin antibodies (C). D, quantification of the results was performed by densitometric analysis, and the percentage (from control untreated cells) of topo I was calculated. The results are means $\pm$ S.D. of four different experiments. E, increasing amounts of nuclear proteins from the aforementioned treatments were added to a topo I reaction mixture, and the reaction products were analyzed as described under *Materials and Methods*.



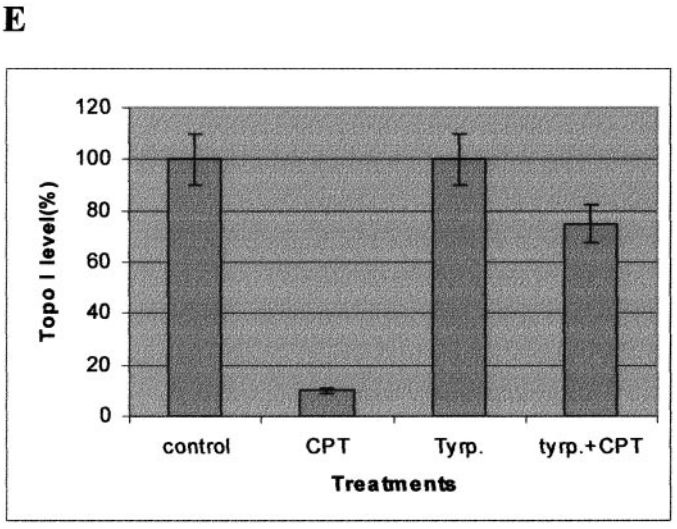


Fig. 5. Tyrphostin treatment reduced the level of CPT-induced DNA-enzyme cleavable complexes. IC9 cells were treated with CPT (60 μM) for 45 min (lane 2), with AG-1387 (240 μM) for 3 h (lane 3), or with treated with AG-1387 followed by CPT treatment for 45 min (lane 4); results from control untreated cells are shown in lane 1. Cells were immediately lysed by SDS, and equivalent volumes (50 μl) were analyzed by Western blot using anti-topo I antibodies (A), anti-topo II antibodies (B), anti-poly-(ADP-ribose) polymerase antibodies (C), or anti-β-actin antibodies (D). Quantification of the results represented in A was performed using densitometric analysis, and the percentage of topo I (from control untreated cells) was calculated. The results are means ± S.D. of four different experiments (E).

tion and prevented the binding of the enzyme to the DNA (Aflalo et al., 1994; Argaman et al., 2003). To further substantiate the notion that topo I is one of the targets of tyrphostin in the cell and to examine its mode of action, we determined the effect of treatment of cells with tyrphostin on the cellular CPT-induced topo I degradation (Desai et al., 1997, 2001, 2003), topo-I /DNA cleavable complexes (Nitiss and Wang, 1996), and topo I-Ub/SUMO conjugates (Desai et al., 1997, 2001; Mao et al., 2000). The results revealed that pretreatment of cells with tyrphostins before the addition of CPT significantly prevented the degradation of topo I protein and reduced the level of the DNA-enzyme cleavable complexes and the formation of topo I conjugates. Because CPT

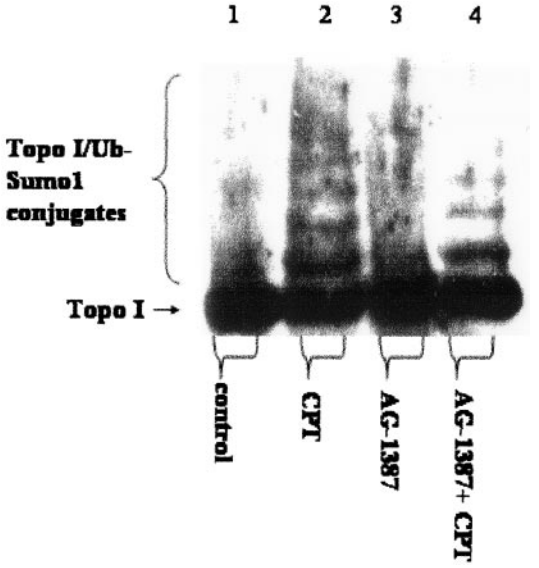


Fig. 6. Tyrphostin treatments reduced the level of CPT induced topo I/Ub-SUMO I conjugates. Cells were treated with CPT for 10 min (lane 2), with AG-1387 for 3 h (lane 3), or with AG-1387 for 3 h followed by CPT for 10 min. Cells were lysed as described under *Materials and Methods*. Equivalent volumes (50 μl) of supernatant were analyzed on 5% polyacrylamide gel electrophoresis. Western blot analysis with anti-topo I antibodies was performed.

inhibits topo I by stabilizing the enzyme-DNA cleavable complexes in interactions with both DNA and the enzyme (Wang et al., 1997), one might suggest that tyrphostin AG-1387 prevents the CPT-induced effects on topo I in the cell by altering the binding ability of this enzyme to the DNA. These data, which are compatible with our previous in vitro results obtained with purified topo I, suggest that topo I is one of the cellular targets of tyrphostin AG-1387. Therefore, tyrphostin derivatives that inhibit essential cellular enzymes, such as topo I and protein tyrosine kinases, may serve as potent anticancer drugs. The efficacy of a combined treatment of tumor cells with tyrphostin and other anti-topo I inhibitors, such as CPT, is not yet clear. However, because pretreatment of tumor cells with tyrphostin, before the addition of CPT, prevented the CPT-induced functions in the cell (especially topo I degradation), these compounds might alter the lethal effect of CPT.

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